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The Specific Mutagenic Effect of Base Analogues on Phage T4

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Several purine and pyrimidine analogues which are sterically very similar to the normal nucleic acid bases have been tested for their mutagenic effect upon T4 phages. Besides 5-bromodeoxyuridine only 2-aminopurine and 2,6-diaminopurine were effective.

Most of the base analogues tested inhibit bacterial growth when present in sufficiently high concentrations, and some of them show an additional effect upon phage growth. Thus 5-bromodeoxyuridine and 5-bromouracil inhibit phage growth (especially in the derivatives of bacteria K-12), and 2-aminopurine depresses lysis inhibition under certain growth conditions.

The "mutability spectrum" of T4 *r*II-type phage mutants induced by 5-bromodeoxyuridine was found to be essentially the same as that of 5-bromouracil-induced mutants studied by Benzer & Freese (1958). In contrast, the mutability spectrum of 2-aminopurine-induced mutants was different from both spontaneous and bromouracil-induced mutants. The genetic coincidence, however, of some 2-aminopurine-induced mutants with some bromouracil-induced mutants indicates some mutagenic similarity between the two base analogues.

The high frequency of phage mutants after growth in the presence of the base analogue is not due to selection of pre-existing mutants, as shown by a fluctuation test. Again, the differences between the mutability spectra are not due to any selection but are caused by preferential mutagenic actions at certain sites of the genome.

1. Introduction

A new method of investigation promises an understanding of mutations in molecular terms. It now seems clear that most hereditary information is carried by deoxyribonucleic acid (DNA) ‡, and that this information is determined by the precise sequence of purine and pyrimidine bases in DNA. Most mutations, therefore, should be due to changes in the base-sequence of DNA or to alterations in the number of DNA molecules. Recently new mutagenic chemicals have been found whose mutagenic reactions with DNA inside the living cell may be analyzed. Especially promising are certain highly mutagenic analogues of the normal DNA bases, since their action may be expected to be simple and rather direct. In addition, genetic fine structure analysis is now possible in several micro-organisms, and this can reveal the mutagenic specificity of a base analogue. The correlation of this genetic specificity with biochemical observations should lead to a clear picture of the underlying mutagenic events.

† Research Fellow of the Damon Runyon Memorial Fund for Cancer Research.

‡ The following abbreviations are used in this paper: AICAR, 5-amino-4-imidazole-carboxamide ribotide; AM, aminopterin; AMP, adenosine monophosphate; AP, 2-aminopurine; BD, 5-bromodeoxyuridine; BU, 5-bromouracil; DNA, deoxyribonucleic acid; GMP, guanosine monophosphate; HMC, 5-hydroxymethyl uracil; IMP, inosine monophosphate.

Dunn & Smith (1954) found that 5-bromouracil (BU) can quantitatively replace thymine in the DNA of phage T2, and Litman & Pardee (1956) showed that BU is also highly mutagenic for these phages. These encouraging biochemical findings stimulated Benzer & Freese (1958) to compare phage T4 mutants induced by BU with those which occur spontaneously, by means of a genetic fine structure analysis. They found that most BU-induced mutations occurred at locations genetically different from most of the spontaneous ones. In addition this analysis revealed a more elaborate mutagenic specificity than any simple model of mutation would have predicted. The mutations of many independently isolated *spontaneous* mutants are not randomly distributed, but recur frequently at certain genetic sites. Such sites of high mutability ("hot spots") have also been found among the *BU-induced* mutants, but their genetic locations are different from the spontaneous ones. The existence of sites of high mutability in itself indicates a specificity of the mutagen, while the difference in location shows that different mutagens have different specificity.

However, a direct correlation of the genetic specificity and the molecular specificity of mutagens cannot be obtained from these observations, especially since the origin of spontaneous mutations is unclear. In an attempt to obtain such a correlation, two approaches have been used in the present work.

(1) The same base analogue, BU, has been applied again for the induction of mutations under different growth conditions to see whether or not the mutagenic specificity changes. No significant change has been observed.

(2) A number of other base analogues which might possibly be incorporated into DNA have been analysed for their mutagenicity. Only two showed a significant mutagenic effect, namely 2-aminopurine (AP) and 2,6-diaminopurine. AP was used to induce a new set of T4 mutants. These exhibited a mutability spectrum (Fig. 2) different from both the spontaneous and the BU spectra, although it had some similarity with the latter.

2. Materials

(a) *Bacteria*

(i) *Derivatives of Escherichia coli B*

B (Benzer), T1 resistant.

BB (Stent), T1 resistant.

B-96 and *B-97* (Gots), both purine-deficient; both were obtained by UV irradiation and penicillin selection from the same *E. coli* B (Gots). *B-96* is blocked in the transformylase reaction between 5-amino-4-imidazole-carboxamide ribotide and inosinic acid and grows on H-medium with supplements of adenine, hypoxanthine, xanthine, or guanine (Gots & Love, 1954). *B-97* is blocked in the desuccinylation of both adenylosuccinic acid ribotide and succinyl-aminoimidazole-carboxamide ribotide, and grows with adenine but with none of the other three bases (Gots & Gollub, 1957).

(ii) *Derivatives of E. coli K12*

S (Benzer); λ-sensitive, T1 resistant.

K (Benzer); lysogenic for prophage λ, T1 resistant.

(b) *Phages*

T4 (Benzer) standard type and several *r*II-type mutants: spontaneous *r*-mutants and 5-bromouracil induced N-mutants.

(c) *Media*

Broth, 1 % bacto-tryptone (Difco) plus 0·5 % NaCl.

Synthetic medium, per 1 liter distilled water: 17·7 g $Na_2HPO_4.12\,H_2O$; 3 g KH_2PO_4; 1 g NH_4Cl; 5 g glucose; 10^{-3} M-$MgSO_4$; 10^{-4} M-$CaCl_2$; 10^{-6} M-$FeCl_3$; 20 mg L-tryptophan,

necessary for phage T4 adsorption and for growth of the T1-resistant bacteria (which are tryptophan-dependent).

H-Medium, synthetic medium plus 20 μg/ml. histidine.

F8 Medium, a synthetic medium which contains per liter: 20 mg L-glycine, 20 mg L-methionine, 10 mg L-leucine, 10 mg L-valine, 10 mg L-serine, 10 mg adenine, 10 mg guanine, 5 mg uracil, 10 mg deoxycytidine, 0·2 mg calcium pantothenate, 0·2 mg thiamine, 0·2 mg pyridoxine, 10 μg vitamin B_{12}.

(d) *Analogues*

Aminopterin (J. M. Ruegsegger, Lederle Medical Research Department, American Cyanamide Company, Pearl River, New York) was kept dark in the cold as a solution of 2 mg/ml. in 1/100 N-NaOH.

2-chloro-6-oxypurine (J. Montgomery, Southern Research Institute, Kettering Meyer Lab., Birmingham, Alabama).

6-methylaminopurine (G. H. Hitchings, Wellcome Research Lab., Tuckahoe 7, New York).

Purine-9-riboside (G. B. Brown, Sloan Kettering Inst. for Cancer Research, New York 21, New York.)

The other base analogues are commercially available (California Foundation, Sigma, Nutritional Biochemicals).

(e) *Plates*

Plates contain either broth or a synthetic medium, plus 1·5 % agar (Difco) with a top layer of the same medium plus 0·7 % agar.

3. Experimental

(a) *Growth of bacteria under conditions of pyrimidine and purine limitation*

The mutagenic effect of base analogues is strongly dependent on the concentration of the natural nucleic acid precursors. With some base analogues any significant mutagenesis can be found only when the formation of the normal base is partially suppressed. In the following we shall describe methods for obtaining limitation in the supply of one or another of the natural pyrimidine or purine nucleotides. It may be recalled that each of the natural nucleotides can be obtained in two different ways, either by *de novo* formation from simple building blocks, or by a "salvage" pathway, using externally provided purines, pyrimidines, or their nucleosides. Generally a limitation of a natural nucleotide will be obtained by inhibiting the corresponding *de novo* pathway and providing only a limited amount of the base which can enter the salvage pathway. We shall describe how the *de novo* pathway of thymine and hydroxymethyl cytosine (HMC) can be inhibited by counteracting folic acid with aminopterin, and how the *de novo* pathway of adenine and guanine can be shut off by using purine-dependent bacteria.

(i) *Bacterial growth in aminopterin-containing medium*

Aminopterin (AM) is a folic acid analogue which inhibits growth and duplication of bacteria *E. coli* B. Webb & Nickerson (1956) showed that it induces the development of long bacterial filaments when certain growth conditions are applied. Such growth conditions are obtained, for example, when bacteria B are grown logarithmically in F8 medium to a titer of 5×10^7/ml. and 50 μg/ml. AM are added. Within 5 hours all bacteria develop into long threads (about 1/50 mm length). Comparison of the microscope count with the colony count on broth plates shows that the bacteria rapidly die under these growth conditions. When the medium also contains 20 μg/ml. (or more) thymine the bacteria develop normally. These observations resemble those of Cohen & Barner (1956) concerning growth in sulfanilamide-containing medium. While sulfanilamide competes with *p*-aminobenzoic acid and thereby prevents the formation of folic acid, aminopterin competes with folic

acid itself. Folic acid is known to be necessary for the methylation and hydroxymethylation of a number of compounds including the *de novo* formation of thymine (Friedkin & Kornberg, 1957). Its partial inhibition therefore results in the inhibition of the methyl transfer and the above observations can be attributed to "thymine-less death".

At higher concentrations of AM, e.g. 200 μg/ml., the bacteria stay small and grow very slowly in F8 and much faster when 20 μg/ml. or more thymine are present. At this AM concentration another metabolite besides thymine is probably partially suppressed and the imbalance between cell surface and cell content is less pronounced.

(ii) *The metabolic block of the purineless bacteria*

The *de novo* pathway of purine compounds goes via 5-amino-4-imidazole-carboxamide ribotide (AICAR) to inosinic acid (IMP) and then branches to give both adenosine monophosphate (AMP) and guanosine monophosphate (GMP) (Buchanan *et al*, 1957). AMP is subsequently used not only for the production of other adenosine nucleotides but also for the formation of histidine (Magasanik, personal communication). AICAR is re-formed as a by-product of histidine synthesis. Externally-given adenine which is converted into AMP by a "salvage pathway" can give rise to the formation of IMP, and thus GMP, by two pathways. On the one hand it can be converted to AICAR and thence to IMP, and on the other hand AMP can be directly deaminated to IMP.

With this knowledge the biochemical blocks of the two purine-less bacterial mutants are easily described. *B-96* is blocked in one of the enzymes that convert AICAR to IMP and therefore can grow on any externally-given purine. *B-97* is blocked in the enzyme which removes succinic acid from purine compounds. This enzyme is used at two places, once in the *de novo* synthesis of AICAR and again in the conversion of IMP to AMP. *B-97* therefore can only grow on externally-given adenine but not on any other of the three purine bases.

It should be mentioned that the amount of histidine or thiamine in the bacterium regulates how much AMP enters the histidine pathway (Magasanik, personal communication). This feedback mechanism is disturbed in purineless bacteria when they are grown in synthetic medium plus adenine alone; the bacterial growth is appreciably inhibited. This inhibition can be removed by the addition of 20 μg/ml. histidine or by 0·25 μg/ml. thiamine. Therefore H-medium was usually used for these bacteria.

(iii) *Bacterial growth in 2-aminopurine-containing medium*

When bacteria *B-96* or *B-97* are grown in H-medium plus 10 μg/ml. adenine and 500 μg/ml. or more AP, *B-96* cells develop into long threads while *B-97* cells divide nearly normally, after some transitory lengthening. The lengthening of *B-96* is more pronounced when 0·25 μg/ml. thiamine replaces the histidine in the medium; after 15 hours practically all of the *B-96* cells are long, up to 0·1 mm, and 97 % are unable to proliferate. The essential difference between the two bacteria, when they are grown in adenine, is that *B-97* can make IMP from AMP using either of the two above-mentioned pathways while *B-96* can do so only by the deamination of AMP. The different effects of AP upon these two bacteria may therefore suggest that AP interferes with the deamination of adenine compounds (e.g. AMP to IMP) and in *B-96* prevents sufficient production of GMP. Indeed *B-96* shows less filament formation when it is grown with AP together with xanthine, hypoxanthine or guanine.

(b) *The mutagenicity of different base analogues for T4 phages*

In order to find another mutagenic base analogue which could possibly be incorporated into DNA, a number of closely related DNA base analogues was tested for their mutagenic effect on phage T4.

The simplest way to detect a large mutagenic effect consists of a spot test for the induction of reversions. Phages of a reverting *r*II-type mutant are plated on a mixture of bacteria K and B, and the base analogue is added to one spot of the plate. An appreciable increase in the number of plaques around the spot can be attributed to the ability of the base analogue to increase the reversion frequency. This spot test has the further advantage that each revertant phage which arises produces only one plaque; the large fluctuation in the number of mutants which one observes in liquid cultures (jackpots) are thereby eliminated. Two reverting *r*II-type mutants

were used, the spontaneous *r*131 and the BU-induced N19. The results, summarized in Table 1, columns 1 and 2, show that 2-aminopurine (AP), 2,6-diaminopurine, 5-bromouracil, and 5-bromodeoxyuridine exhibited a mutagenic effect for the reversion of N19, while no increase in plaque numbers was observed with the mutant *r*131. No normal DNA bases, or their deoxyribonucleosides, had any effect.

TABLE 1

Effect of different base analogues upon bacteria and phages

Mutagenic effect upon phages: 2×10^7 phages T4 N19 (*r*II-type mutant, induced by 5-bromouracil, spontaneous reversion index = $0{\cdot}05 \times 10^{-6}$) and T4 *r*131 (*r*II-type spontaneous mutant, spontaneous reversion index = $0{\cdot}5 \times 10^{-6}$) were each plated on broth with 2×10^8 bacteria K and 2×10^7 bacteria B. Some of the base analogue was placed in powder form at the center of each plate, which was then immediately put into a refrigerator for 3 hr in order to permit diffusion. During the subsequent over-night incubation at 37°C all phages infecting B are able to multiply and to undergo mutations. When all bacteria B are destroyed by lysis only the phage revertants can continue to multiply on K and give rise to a plaque. The test is positive if many plaques appear in the neighbourhood of the spotted base analogue.

Effect upon bacteria and plaques: 200 phages (standard, or one of the *r* types) were plated with 2×10^8 bacteria on broth, and the base analogue was placed in powder form in the center of the plate. After 12 hr diffusion time in the cold the plate was incubated overnight at 37°C. For the bacterial inhibition: — = no effect observed, + = little inhibition, ++ = strong inhibition.

Base analogue	1 Induction of reversions for the T4 *r*II-type phages		3 Inhibition of bacteria	4 Effect on T4 phages
	*r*131	N19		
2-aminopurine	—	++	+	standard type gives large plaques on B, BB. No effect on S, K.
2-amino-6-oxypyrimidine	—	—	—	none
2-amino-6-thiopurine	—	—	—	none
8-azaguanine	—	—	+	none
6-azathymine	—	—	+	none
2-chloro-6-hydroxypurine	—	—	+	none
2,6-diaminopurine	—	+	++	none
5-hydroxymethylcytosine	—	—	—	none
6-mercaptopurine	—	—	+	none
6-methyladenine	—	—	++	none
5-methylcytosine	—	—	—	none
6-methylpurine	—	—	++	none
purine	—	—	++	none
purine-9-riboside	—	—	++	inhibits slightly
2-thioadenine	—	—	—	none
5-bromouracil	—	+	+	inhibits
5-bromo-deoxyuridine	—	++	+	inhibits slightly
2-thiocytosine	—	—	—	none

In this reversion test only those mutations can be detected which restore the ability of the genome to make a functioning protein (enzyme). For the *r*II locus these reversions do not seem to arise as the result of a "suppressor mutation" at another locus, because the existence of several non-reverting "point" mutations (see Fig. 2) makes such a suppressor effect improbable and because crosses between some revertants and standard type phages have shown that the reversion involves a mutation closely linked to, or at, the original mutational site. Thus only mutations at one or a very small number of genetic sites within the *r*II region will be able to cause the reversion of a given mutation.

The reversion test for a given *r*II mutant may therefore reveal the mutagenicity of *some* mutagens by the induction of reversions, and be inert to others. Such a specificity of different mutable sites is demonstrated in the foregoing test, in which phage N19, but not *r*131, responded to base analogues. The mutagenic response should be much less specific if the induction of *forward* mutations is measured. For in this case changes at any one DNA site necessary for a functioning protein can be detected. The number of these indispensable sites is large and at least some of them should respond to the mutagen.

Therefore all the purine analogues were tested once more, this time for the induction of forward mutations under partial purine deprivation. The purine-less bacteria *B-96* were grown and infected under conditions (described in a later section) which give a strong mutagenic effect of 2-aminopurine. The base analogue was added in amounts of 100 μg/ml. and of 500 μg/ml. A significant mutagenic effect was found only for AP and 2,6-diamino-purine. In repeated tests between 0·5 % and 1·5 % *r*-mutants were produced.

The inhibition of bacterial growth, displayed on plates by a less turbid or even clear circle around the base analogue, is qualitatively summarized in Table 1, column 3. Most of the analogues inhibit the bacterial growth more or less.

(c) *The effect of different base analogues on T4 phage growth*

In addition to the inhibition of bacterial growth, some base analogues also affect normal phage growth. This additional effect can be observed, for example, in phage platings, beyond the area in which the base analogue spot exerts a strong bacterial inhibition. Table 1, column 4 summarizes the results. Of the base analogues used, only purine 9-riboside, 5-bromouracil (BU), 5-bromodeoxyuridine (BD), and 2-amino purine (AP) showed such an additional effect; it will be described in somewhat more detail, as far as this seems necessary for the study of the mutagenic effect of these base analogues.

Both BU and BD inhibit phage growth on plates strongly in bacteria S and K, and little in B. For smaller concentrations of BD (50 μg/ml.) the mutagenic effect is dominant while for higher concentrations the inhibition becomes more effective. When phages are grown in broth, BU shows a stronger inhibitory and a much weaker mutagenic effect than does BD.

AP depresses lysis inhibition under certain growth conditions. This can be seen most simply when *r*- and standard-type T4 phages are plated together with B on broth, or with *B-96* or *B-97* on synthetic medium plus 0·25 μg/ml. thiamine and 10 μg/ml. adenine. When 10 mg or more AP is added to the top layer the standard type phages produce plaques which are nearly (B) or completely indistinguishable from those of the *r*-type phages. Simultaneously, all "plaque interaction" between *r*- and standard-type phages vanishes. These large plaques, to which a standard-type

phage gives rise, are not caused by a selection of *r*-mutants, since upon isolating and replating the phages with B on broth, most of them (about 95 %) again give standard-type plaques. The effect of AP is counteracted completely by the addition of adenine in the same concentration.

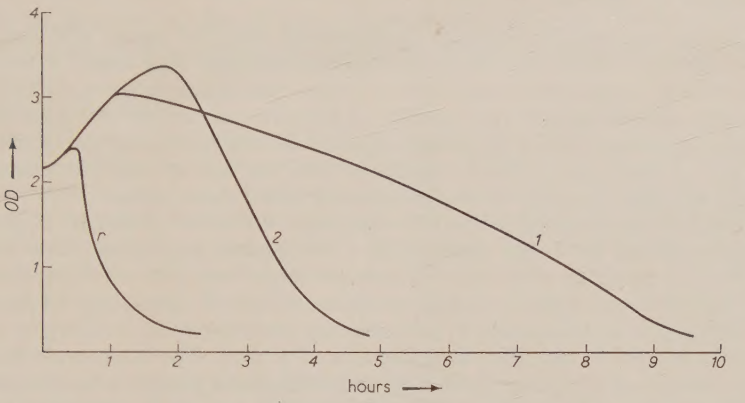


FIG. 1. *Lysis inhibition under different growth conditions. First infection with r-phage:* rapid lysis (curve *r*) independent of medium. *First infection with standard type phage:* B-97 with thiamine, histidine, or both: curve 1. B-97 with same, plus AP: curve 2. With histidine alone the decrease is less rapid than with thiamine. B-96 with histidine: decrease slower than in curve 1. AP only slightly effective. B-96 with thiamine, or both: curve 2. AP only slightly effective.

The effect of AP upon lysis inhibition in liquid medium is summarized in Fig. 1. For bacteria *B-96* the presence of thiamine alone reduces lysis inhibition and AP is only slightly effective at the concentrations used. For bacteria *B-97*, however, AP markedly accelerates lysis.

The measurements summarized in Fig. 1 were done under the following conditions. Bacteria were grown overnight in synthetic medium plus 40 μg/ml. adenine and either 20 μg/ml. histidine, 0·25 μg/ml. thiamine, or both. They were resuspended in the same medium, but with 10 μg/ml. adenine, at a concentration of 10^8/ml. and grown to 2×10^8/ml. After chilling, T4 standard type phages were added to give a multiplicity 2 and AP to give 500 μg/ml. After 8 min adsorption the culture was aerated at 37°C and 15 min later superinfected with *r*II-type phages at a multiplicity 10. The development of lysis was recorded by measuring the optical density at 600 mμ, calling O the time of incubation at 37°C. The curves given are typical for the *relative* differences under different growth conditions. The *quantitative* time-course of lysis depends on the concentration of the bacteria and of the residual adenine in the medium.

(d) *The mutability spectrum of rII-type mutants induced by 5-bromodeoxyuridine*

A mutability spectrum of T4 *r*II-type mutants included by BU has been reported by Benzer & Freese (1958). These mutants had been induced in a sulfanilamide-containing medium in which the formation of folic acid was suppressed. Flaks & Cohen (1957) have shown that bacteria B, after infection with T6 phage, synthesize an enzyme which converts deoxycytidylic acid into 5-hydroxymethyldeoxycytidylic acid and which requires tetrahydrofolic acid as a cofactor. It may therefore be presumed that sulfanilamide depressed the synthesis not only of thymidylic acid but also of HMC. We wanted to know whether the same or a different spectrum would be produced under different growth conditions using a medium in which folic acid was again counteracted, but to which excess thymidine was added to depress the incorporation of bromouracil into DNA. The addition of thymidine should not influence HMC

formation since Cohen (1956) has shown that the methyl groups of thymine and methionine are not converted to the hydroxymethyl group of HMC. The mutants were induced by BD in a synthetic medium, using aminopterin as a folic acid antagonist, and thymidine was added to give a 4 : 1 ratio of thymidine to BD.

(i) *Production of mutants*

E. coli B was grown in F8 medium supplemented with 200 μg/ml. AM and 200 μg/ml. thymidine. In this medium the bacteria duplicate and stay very small. When they were grown for 6 hr to a concentration of $1{\cdot}5 \times 10^8$/ml. and singly infected with T4 standard type phages (multiplicity 10^{-3}) the number of infective centres (plus unadsorbed phages) decreased to about one half of its initial value by 30 min after infection. Some lysis then occurred and a burst size of 20–30 resulted. Both the loss of infective centres and the small burst size may possibly be due to a partial HMC deprivation.

BD mutants were produced in the following way: 0·1 ml. of overnight B, grown in F8, was added to 30 ml. F8 + 200 μg/ml. AM + 200 μg/ml. thymidine. The culture was aerated for 7 hr at 37°C, when the microscope count was $2{\cdot}3 \times 10^8$/ml. (viable count $2{\cdot}2 \times 10^8$/ml.). BD was added to a final concentration of 50 μg/ml. and T4 standard type phage added to give a multiplicity 2. Immediately after infection one drop of the mixture was delivered to each of 180 tubes standing in a 37°C bath. The tubes were incubated for $8\frac{1}{2}$ hr; then broth was added and each tube was diluted and plated on broth plates with B. After incubation at 37°C one *r*-mutant was picked from each plate on which *r*-mutants were found, and replated with B. From each new plate one plaque was picked and a phage stock grown with bacteria BB. The remaining undistributed mixture was aerated for 11 hr and some drops of chloroform were added to induce lysis; the lysate was plated on B for the determination of phage titer and *r*-mutant frequency. It showed a 20-fold increase of phage titer over the input and contained 1 % *r*-mutants. In the same medium without BD, the frequency of *r*-mutants was about 0·1 % which is higher than the usual "spontaneous" background.

(ii) *Genetic mapping of BD-induced mutants*

The series of independently arising *r*-type mutants were numbered N171 through N343. 103 of these were of the *r*II-type, the rest *r*I or *r*III. Each of the *r*II-type mutants could be assigned to one of the segments into which the *r*II region can be subdivided by means of mutants containing large alterations (see Benzer & Freese, 1958, and Fig. 2). Further mapping could be achieved for all but three very leaky mutants. This mapping was done relative to the genetic location of all former BU-induced mutants and of all those spontaneous mutants which were available, and also with respect to all AP-mutants to be described later.

Most of the crosses were made by means of a simple spot test described previously (Benzer & Freese, 1958). About 2×10^7 phages of one mutant type were plated on a mixture of bacteria K and B (ratio 20 : 1, or 100 : 1 for leaky mutants). Phages of the other mutant type were placed on this plate as a small drop or streak as soon after plating as possible; this spot has to contain sufficient phage for recombination but not too many revertants whose frequency is checked on a control plate.

The spectrum of mutations induced under these conditions is shown in Fig. 2 (second horizontal line). Since the background of spontaneous mutants was higher than for the BU-induced spectrum of Benzer & Freese (1958) (Fig. 2, third horizontal line) it is not surprising that some mutants have the genetic location and reversion properties of the spontaneous hot spots *r*131 and *r*117. But otherwise it is obvious that essentially the same mutability spectrum has been reproduced as was reported for BU-induced mutants, with a reasonable variation in the number of mutants at each genetic site. Especially noteworthy is the recurrence of the hot spots N11, N12, and N24, and also the large number of mutants at site N29. Nearly all of the mutants revert. Only one non-reverting mutant has been found at site N29.

(e) *The mutability spectrum of rII-type mutants induced by 2-amino purine*

To obtain conditions under which the mutagenic effect is high, and therefore the background of spontaneous mutants low, the purine-less bacteria *B-96* and *B-97* were used, since AP is a purine analogue. Under complete purine starvation (*B-96* or *B-97* grown exponentially in H-medium plus 25 μg/ml. adenine, then washed and resuspended in H-medium) the phage mutation-rate did not increase appreciably. Some adenine was therefore added and a strong mutagenic effect was found.

(i) *Production of mutants*

An exponentially-growing culture of *B-96* (in H-medium plus 25 μg/ml. adenine hemisulfate) was diluted to give a microscope count of 5×10^7 cells/ml. in an H-medium with the final concentration of 10 μg/ml. adenine and 500 μg/ml. AP. The bacteria were grown for 4 hr at 37°C at which time the microscope count was about $1{\cdot}2 \times 10^8$ cells/ml. (cells were about three times as long as normally). T4 standard type phages were then added to give 10^7 phages/ml., the culture was aerated for 10 hr at 37°C, and then chloroform was added. The phage titer was 3×10^9/ml. (plated on B). These phages contained many plaque-type mutants, among which *r*-mutants occurred with a frequency between 1 and 2 %. In controls, grown with the same amount of histidine and adenine, but without AP, the frequency of *r*-mutants was normally less than 0·1 %.

The same mutagenic effect was obtained with the adenine-specific purine-less mutant *B-97*, when grown under similar conditions. This shows that the specific inhibition, observed with *B-96* and described above, has no influence upon the mutation rate.

The mutability spectrum of AP-induced mutants was determined twice, once using phage mutants arising in bacteria *B-96* and once using mutants arising in *B-97*. The bacteria grown under the conditions described above were singly infected with standard type phages (50 to 80 phages/ml.), immediately distributed by drops to many tubes, and 60 min after infection plated with bacteria B. From each plate only one *r*-type mutant was picked and purified by replating, and thus a large number of independent mutants was isolated, which were numbered AP1, AP2 and so on.

(ii) *Genetic mapping of AP mutants*

Of 156 such *r*-type mutants arising in *B-96*, 53 were of the *r*II-type; and of the 144 *r*-type mutants arising in *B-97*, 47 were of the *r*II-type. Each of the *r*II-type mutants could be assigned to one of the *r*II-segments by the spot test technique. The further mapping of the AP-mutants with respect to one another, to the bromouracil (or bromodeoxyuridine) induced N-mutants, and to the 25 available spontaneous *r*-mutants, could be achieved for all but four leaky mutants of the *B-96* set and one leaky mutant of the *B-97* set. The results are shown in Fig. 2 (fourth and fifth horizontal lines).

Many of the phage AP-mutants induced in bacteria *B-96* coincide genetically with those induced in *B-97*. At certain sites mutants recur frequently in both sets, and the variation of their number is within reasonable limits. Hence, the mutability spectra of AP mutants induced into the bacteria *B-96* and *B-97* are essentially alike, and apparently are not influenced by the difference in bacterial inhibition reported for AP earlier in this paper.

Only a small number of AP-mutants appeared at the genetic location of some spontaneous mutants; most of these have quite a different reversion index indicating a different type of mutation. The two real coincidences can be attributed to a background of spontaneous mutants among the AP-induced ones. Apparently the mutants induced by AP are of a type quite different from most spontaneous mutants.

Finally, a comparison between the AP- and the N-mutants shows again a clear difference in the mutagenic specificity of AP and BU. This is exhibited especially

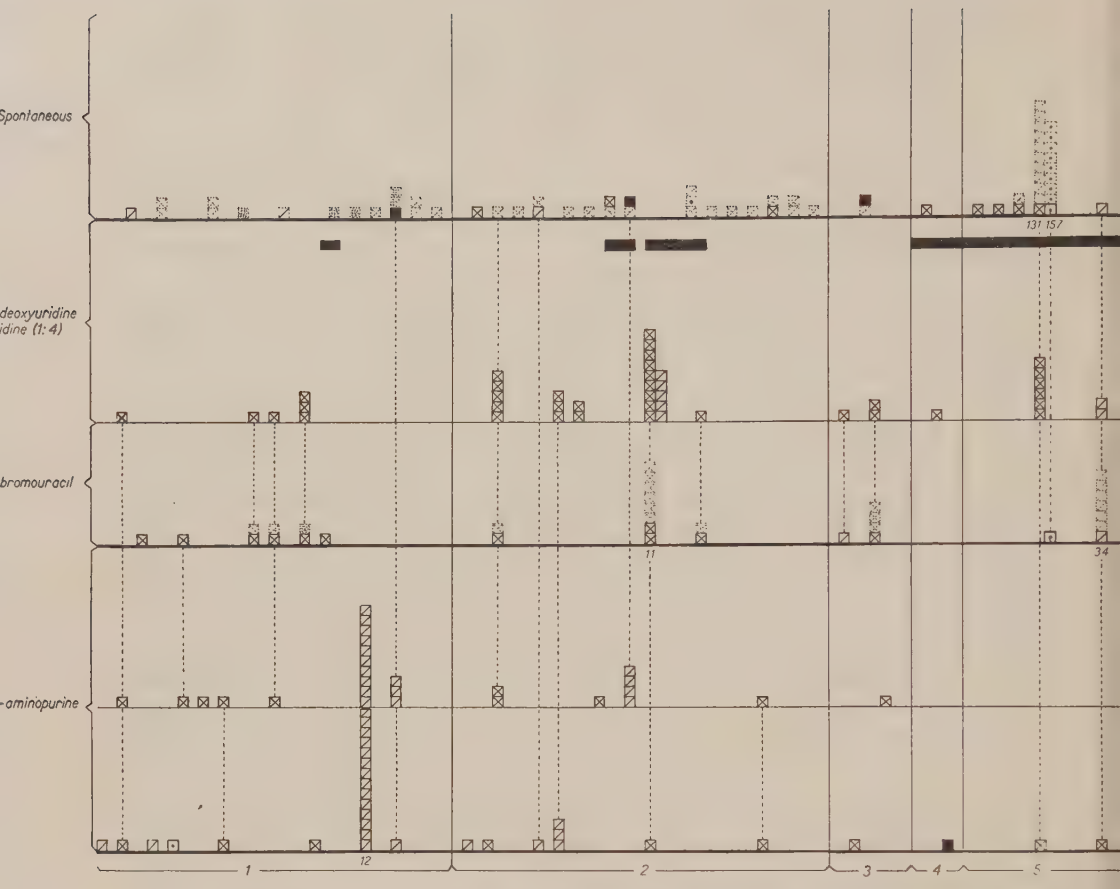


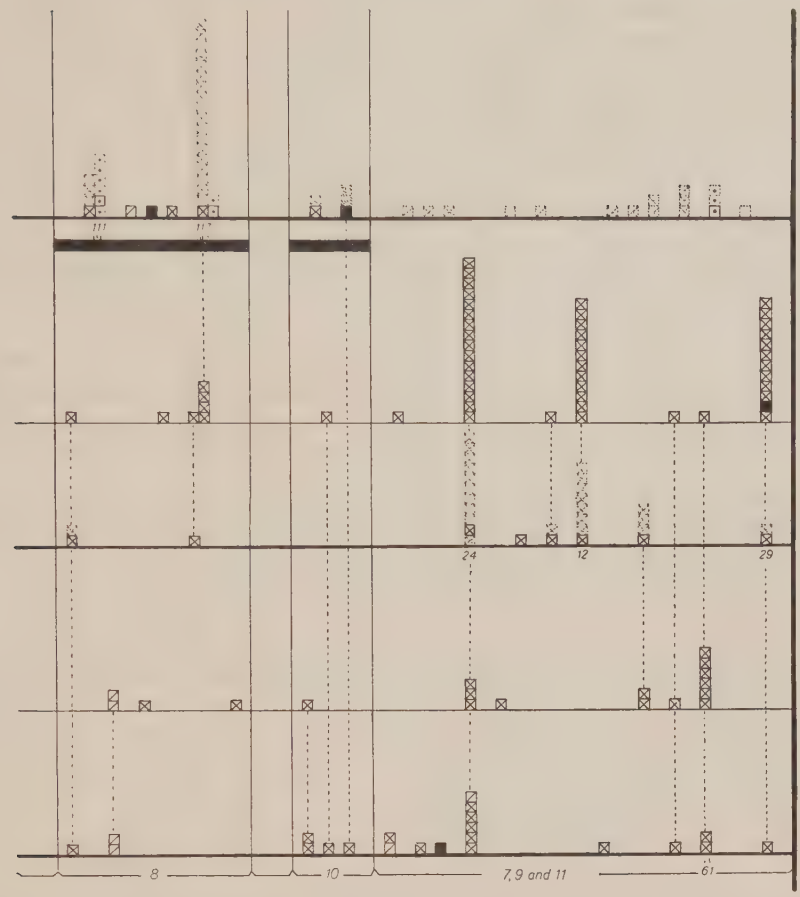
FIG. 2. Genetic map of the *r*II region of phage T4 showing the locations of mutations for independently isolated *r*II-type mutants.

The mutations induced by three different means are vertically displaced: first horizontal line: spontaneous mutations (*r*) (see Benzer & Freese, 1958); second horizontal line: mutations induced by 5-bromodeoxyuridine with the addition of four times as much thymidine (N); third horizontal line: mutations induced by 5-bromouracil (N) (see Benzer & Freese, 1958); fourth horizontal line: mutations induced by 2-aminopurine in bacteria B-97 (AP); fifth horizontal line: mutations induced by 2-aminopurine in bacteria B-96 (AP).

The *r*II region is divided into cistron A and B, and into segments 1, 2, 3, 4, 5, 6, 8; segments 7, 9, 11 are lumped together. Each mutant is represented by a box and placed in the proper segment. Mutants were assigned to a given segment by crossing each to a set of 6 large alteration mutants (see Benzer & Freese, 1958). Mostly by spot tests, and sometimes by normal crosses using a mixed infection of bacteria B, it was determined which mutants of the same segment recombine with one another and which do not. All mutants for which clearly no recombination was found are stacked at the same abscissa of the drawing. Mutants which certainly differ in

well for the hot spot AP12, at which no BU-induced mutant has been found, and for the hot spot N12 at which no AP-induced mutant has been observed.

But the difference in mutagenic specificity between BU- and AP-induced mutants is certainly less than that between induced and spontaneous mutants. As a matter of fact quite a number of AP- and N-mutants coincide genetically; e.g., at the BU hot spot N24 nine AP-mutants have been observed. This shows that some of the sites which can be mutated by BU can also be mutated by AP, while for other sites the two base analogues behave rather differently.



their reversion properties but for which it has not been clearly decided whether or not they can recombine, are shown immediately adjacent to one another. All other horizontally-displaced mutants do recombine. The order and position of recombining mutants within one segment have not been determined. One N-mutant was available for each genetic site at which N mutants have been reported in the previous work by Benzer & Freese (1958), while of the *r*-mutants only some, including all hot spots, were available for this work. All mutants which were not available for crossing are indicated by dotted lines.

The reversion index of each mutant is indicated by shading. A dot ⊡ corresponds to a reversion index larger than 100×10^{-6}; a bar ▨ corresponds to a reversion index between 1 and 100×10^{-6}; a cross ⊠ corresponds to any observed reversion with an index smaller than 1×10^{-6}; and a black box ■ indicates mutants for which no reversion has been observed. The sensitivity of detection of rare spontaneous reversions can be greatly increased when the phages are plated together with a mixture of bacteria K and B (ratio 20 : 1, for example). Applying this technique the rare reversion of some mutants was detected which had not shown any revertants in earlier work (Benzer & Freese, 1958).

Most AP-induced mutants revert, with only two exceptions among 106 mutants. Most of the reversion indices are in the neighbourhood of 10^{-6} (e.g., the hot spot AP12) or smaller.

(f) *Rejection of selection as a cause for the specific mutagenic effect*

Selection could play a role at three different stages of phage-mutant production: (i) The base analogue could select certain *r*-mutants present in the wild type stock, or (ii) it could select for or against certain *r*-type mutants in the bacteria in which

they have been produced, or finally (iii) a selection could take place on the plate by preferential picking of certain phenotypes of *r*-type mutants. None of the three types of selection can be the cause either for the high frequency of *r*-type mutants, or for the difference in mutability spectra.

(i) The high selection of any *r*-type mutants preexisting in the wild type stock can best be excluded by a fluctuation test. For BU such a test has been reported by Litman & Pardee (1956) and it is therefore only necessary to give the similar results for AP. The bacteria *B-96* were grown under the conditions given above for a large mutagenic effect. They were singly infected with T4 standard-type phages and immediately distributed by drops into small test tubes. Each tube received about four phages. After an incubation time of 60 min in a 37°C waterbath all tubes were transferred to a 45°C bath. Soft agar and bacteria B were added to each tube, and the contents plated on broth plates. Each plate contained, on the average, 600 phages, the numbers varying between 70 and 800. (One of the 89 plates tested had no plaques.) Most plaques were of standard type. Among other mutants, varying numbers of *r*- and mottled plaques were found. The arithmetical mean value for the frequency of *r*- and mottled plaques taken together was 1·6 %. The number of plates falling into a given range of percent mutants per plate are given in Table 2. If the large

Table 2

Variation in the number of mutants arising under the action of 2-aminopurine

r- and (clearly) mottled plaques are both counted as mutants. Bacteria singly infected (multiplicity 4×10^{-7}).

Average number of plaques per plate about 600.

Percentage of *r* plus mottled plaques per plate	Number of plates
0	2
$0 < \ldots \leq 0{\cdot}5$	12
$0{\cdot}5 < \ldots \leq 1$	28
$1 < \ldots \leq 2$	26
$2 < \ldots \leq 3$	11
$3 < \ldots \leq 7$	9
11	1

percentage of *r*-mutants among the progeny phages had arisen by selection for *r*, one would expect a very small number of plates with a huge percentage of *r*-mutants, while all other plates would display only standard-type plaques. This follows from the small frequency of *r*-mutants among the infecting standard-type phages (less than 10^{-4}) and the small probability that an *r*-mutant appears spontaneously among the duplicating phages (also less than 10^{-4} per phage per duplication). Only 2 of the 88 plates had no *r*-mutant or mottled plaques and the largest percentage of *r*-plaques found on just one plate was 11 %. This clearly excludes selection.

(ii) Some of the *r*II mutants induced by the base analogue could possibly replicate faster than others, under the action of the base analogue (or the growth medium).

Another analogue (or growth medium) could possibly stimulate or suppress the growth of other types of *r*II-mutants, and thus produce the difference between the mutability spectra. *A priori* this possibility appears rather remote. Each of the *r*II-mutants of the same cistron probably fails to induce the formation of one and the same functioning enzyme, and it seems unlikely that different base analogues could differentiate between such differences by selective inhibition. However, this possible mechanism has also been excluded experimentally. Bacteria were grown under conditions of strong phage-mutagenic effect produced either by BD or by AP. They were then infected with different phage mixtures in different multiplicities to test for selection. The phages used were of three types—standard type, BU hot spot N12, and AP hot spot AP12. The results are summarized in Table 3. For mixed infection

TABLE 3

Effect of different mutagenic growth conditions upon the growth of different phage mutants

The growth conditions were those of high 5-bromodeoxyuridine mutagenicity and of high AP mutagenicity (bacteria *B-96*) as described in the text. The phages were absorbed to bacteria of titer 2×10^8 and the culture aerated at 37°C for 5 hr. After addition of chloroform the phages were assayed on B. *a* and *b* are two separate experiments with different concentrations of BD (0·1 and 0·2 mg/ml.) and AP (0·5 and 1 mg/ml.). r^+ = T4-standard type; N12 = *r*II-type mutant of a 5-bromouracil-induced hot spot; AP12 = *r*II-type mutant of a 2-aminopurine-induced hot spot.

Phages	Plaque types		In stock	After growth in BD		After growth in AP	
				a	*b*	*a*	*b*
N12 + standard	ratio $r : r^+$	=	1·8	1·2	1·4	1·8	1·5
	multiplicity of infection	=		6	6	6	6
				a	*b*	*a*	*b*
AP12 + standard	ratio $r : r^+$	=	1·6	1·1	1·1	1·4	1·4
	multiplicity of infection	=		5	5	5	5
			% of total	plaques	% of total	plaques	% of total
N12 + AP12	N12	=	0·55	67	0·52	70	0·52
	AP12	=	0·45	52	0·40	59	0·44
	r^+	=	0	5	(0·04)	3	(0·015)
	possible double *r* multiplicity of infection	=	0	5	(0·04)	1	(0·02)
		=		1		1	

with *r*- and standard-type the progeny phage have in most cases a slightly higher percentage of standard-type phage than the input (probably due to lysis inhibition); but no strong selection can be observed which is specific for or against a certain *r*II-type mutant and which depends on the medium. The absence of selection is especially clear for the case of infection with the two *r*II-type phages N12 and AP12: The progeny phages were plated on B, all plaques were picked, replated on K + 1/100 B, and the plates were spotted with two large alteration mutants (of cistron A and B) to determine whether they were AP12 or N12. The phage ratios after growth in BD and AP were alike (see Table 3). These results exclude any selection between mutants of a BU and an AP hot spot, under any one of the mutagenic growth conditions used.

(iii) The final possibility is that certain plaque types are picked preferentially. A slight difference in plaque type can sometimes be observed on B between *r*I- and *r*II type mutants, the former tending to be larger with more halo. But among the different *r*II-type mutants no systematic difference has been seen, and hot spot and non-hot spot *r*II mutants could not be distinguished in mixed platings. Nevertheless, to avoid any unconscious selection, the first *r*-type mutant seen on a plate was picked. Apart from these precautions, the experimental evidence clearly excludes the preferential picking of certain mutants on any large scale. Either one would expect to pick the same type of plaque preferentially on every occasion, which should give rise to the same spectrum for BU and AP; or one might select a different plaque type in each experiment in which mutants are isolated, which then should yield a different spectrum each time. The evidence rules out both these alternatives.

It therefore seems to be a valid conclusion that the three different mutability spectra (spontaneous, BU or BD, and AP-induced) are caused by a difference in the mutagenic specificity of the agents responsible for the induction of the mutations, and cannot be attributed to any kind of selection.

4. Discussion

We discuss first the genetic and then the biochemical aspect of this work. The genetic analysis of the *r*II-type mutants induced by 5-bromouracil (BU) or 5-bromodeoxyuridine (BD), and by 2-aminopurine (AP), showed each of the corresponding mutations to be localized in a very small area of the genome. Since nearly all of these mutants revert, perhaps most of them involve the change of just one nucleotide pair in the DNA.

The large number of sites at which mutations are observable are distributed over the whole of the *r*II region, as can be seen in Fig. 2. A given base analogue mutates different sites of the genome with different frequencies, which is strikingly displayed in sites of high mutability ("hot spots"). BU (or BD) and AP exhibit a difference in their mutagenic specificity, for some of their hot spots are at genetically different locations. In contrast to this difference between different analogues the same analogue gave the same mutability spectrum when applied twice under different growth conditions. These observations, in addition to mixed infection experiments and the fluctuation test, prove that the hot spots (and mutability spectra) are caused by specific mutagenesis of the base analogue at certain sites of the genome and not by any kind of selection.

Some of the BU-induced mutations coincide with some of the AP-induced ones, and thus reveal some common specificity. In contrast, practically all the mutations arising in the presence of BU or AP are different from the spontaneous ones, apart from a background of spontaneous mutants. This apparent mutual exclusion may divide the mutable sites into two or more classes so that a given base analogue can mutate the different sites of one class with varying frequency but never those of another class.

We shall now discuss the biochemical aspect and show how the two base analogues may exert their strong mutagenic effect. We first note that, since both analogues can form two hydrogen bonds to one of the normal DNA bases, their incorporation into DNA is structurally possible. BU can pair with adenine and thus replace thymine while AP can pair with thymine and replace adenine (Fig. 3). BU can quantitatively

replace thymine in phage DNA, as shown by Dunn & Smith (1954); the incorporation of AP is being examined.

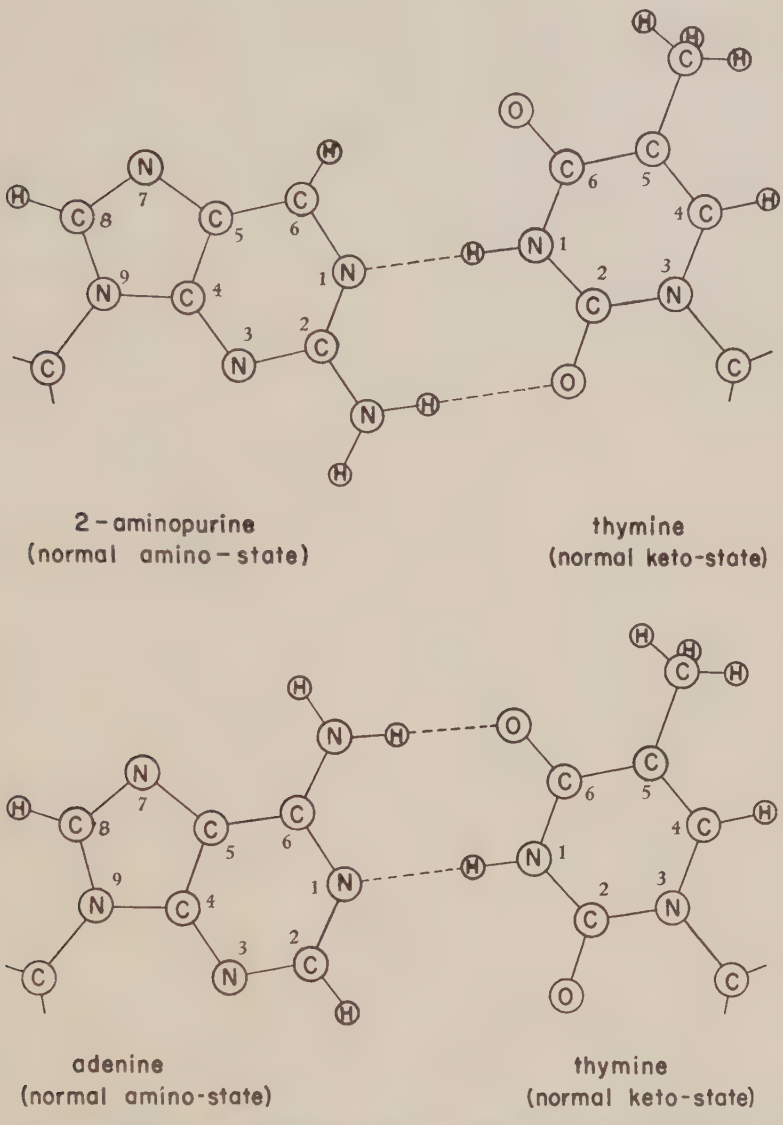


FIG. 3. *Pairing by means of two hydrogen bonds between 2-aminopurine and thymine, as compared to pairing between adenine and thymine.* While the latter pair uses the 1- and 6-positions of the purine and pyrimidine rings, the former occupies the 1- and 2-positions. In 5-bromouracil the methyl group of thymine is replaced by the equally large bromine atom, which has the same van der Waals radius.

It is not experimentally known whether the mutagenic action of BU or AP is direct, by incorporation into DNA, or indirect, by unbalancing the nucleic acid metabolism in the bacterial cell. But the fact that many base analogues which do not seem to be incorporated into DNA (6-mercaptopurine, etc.) inhibit bacterial growth, and yet are not highly mutagenic, makes it probable that the effect of the two base analogues used here is a direct one. We shall therefore discuss how such a direct mutagenic effect may come about using the further assumption that in DNA

replication each new DNA chain is formed along a pre-existing complementary chain (e.g., according to the model proposed by Watson & Crick (1953)).

In this picture permanent mutations are not caused merely by the replacement of a DNA base by its base analogue. For, in subsequent DNA replications in a normal medium, the incorporated analogue can attach to it the base it originally paired with, and thus cause the formation of new DNA with unaltered nucleotide pairs. For instance many of the phages in which thymine has been quantitatively replaced by BU grow as well as normal phages, and 90 % of the growing ones do not exhibit any mutation. Mutations are rather induced by mistakes in base pairing which the analogue may undergo more or less frequently. We shall first examine how such mistakes in pairing can come about and then see how they may induce the transition of one nucleotide pair into another.

The possibility of rare mistaken pairings of normal DNA bases, due to tautomeric shift, has been mentioned by Watson & Crick (1953). BU should behave rather

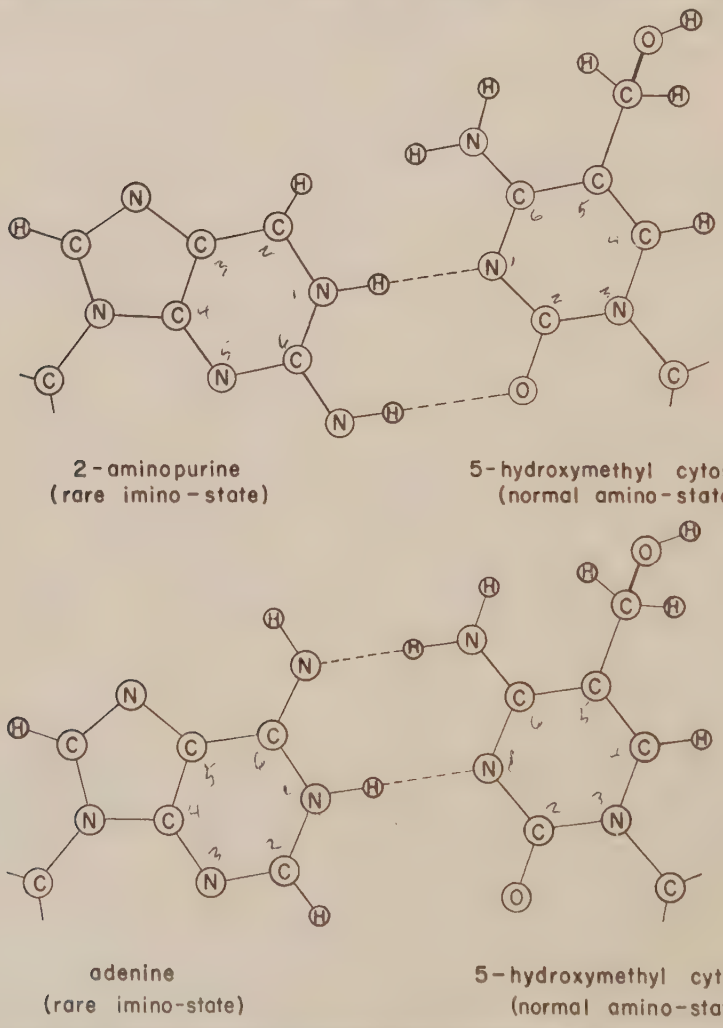


FIG. 4. *The rare "mistake pairing", by means of two hydrogen bonds, between 2-aminopurine and 5-hydroxymethylcytosine (HMC) as compared to the pairing between adenine and HMC.* For both of these pairings a tautomeric shift from the amino- into the imino-state is necessary in one of the bases.

similarly to thymine, and should therefore be able to pair by mistake with guanine. Meselson (personal communication) has pointed out that the higher electro-negativity of bromine (as compared to the 5-methyl group) should increase the frequency of tautomeric shifts in BU over that in thymine; and possibly just the loss of the hydrogen atom in the 1-position of BU could in itself give rise to a mistaken pairing by one hydrogen bond.

AP can make two hydrogen bonds with 5-hydroxymethyl cytosine (HMC), after it has undergone a tautomeric shift (Fig. 4); in this respect it is similar to adenine (Fig. 4). But in contrast to adenine, AP can make one hydrogen bond to HMC even when both bases are in their normal (amino) state (Fig. 5). This one bond alone, since no other groups are sterically hindering, may be sufficient to cause frequent pairing of AP with HMC (or another cytosine base). If this hypothesis is correct, all those 2-aminopurines should be mutagenic which bear a monovalent group at the 6-position, as long as the enzyme system can still convert them into the direct DNA precursor, and the pairing is not interfered with by steric hindrance. It might further be expected that the pairing mistakes of AP would be more frequent than those of BU, since they can occur when AP is in its normal state.

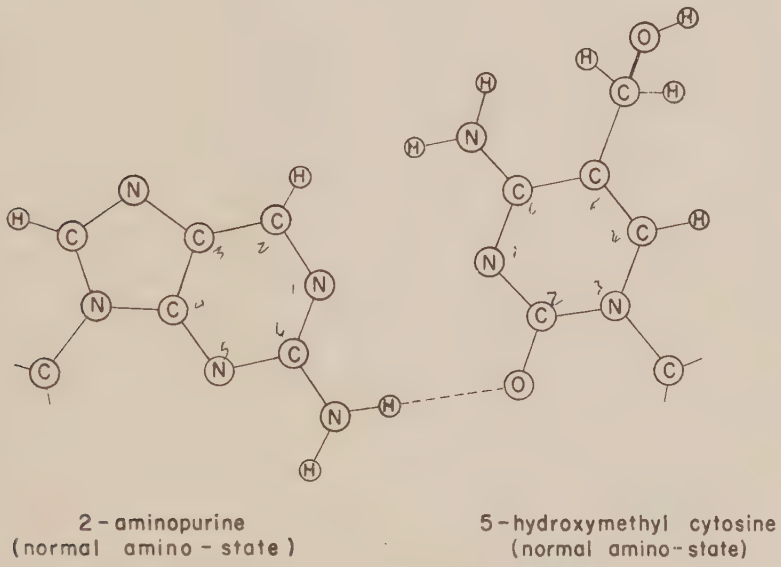


FIG. 5. *The frequent "mistake pairing", by means of one hydrogen bond, between 2-aminopurine and HMC.* Both bases are in their normal amino-state. If this pairing were often fixed in duplicating DNA a high mutation rate would be expected, even under conditions of little incorporation of 2-aminopurine into DNA. Adenine cannot undergo such a frequent mistake-pairing since the two amino groups in the 6-position cannot form any strong hydrogen bonds when they are in the amino-state, nor are any other hydrogen bonds then possible.

Each pairing mistake fixed in replicating DNA by the incorporation of the attaching nucleotide ultimately gives rise to a change of one nucleotide pair in some of the progeny DNA molecules. To understand this clearly, we must consider two distinct possibilities: "mistakes in incorporation" and "mistakes in replication" (see Fig. 6). For purposes of illustration we shall take the case of BU.

A "mistake in incorporation" occurs when the DNA precursor of BU attaches to a guanine nucleotide of a pre-existing DNA chain, so that BU is incorporated in the

place of HMC. In the next DNA replication the incorporated BU attaches to it adenine, its normal complementary base; and in subsequent DNA replications BU can be replaced by thymine, its normal analogue. Ultimately, therefore, an HMC-guanine pair has been replaced by a thymine adenine pair in some of the progeny DNA molecules.

A "mistake in replication," on the other hand, may occur only *after* BU has been incorporated into DNA in the place of its normal analogous base thymine. When this DNA molecule replicates the incorporated BU may by mistake attach a guanine. This ultimately results in the change of a thymine-adenine pair into an HMC-guanine pair in some of the progeny DNA molecules (see Fig. 6).

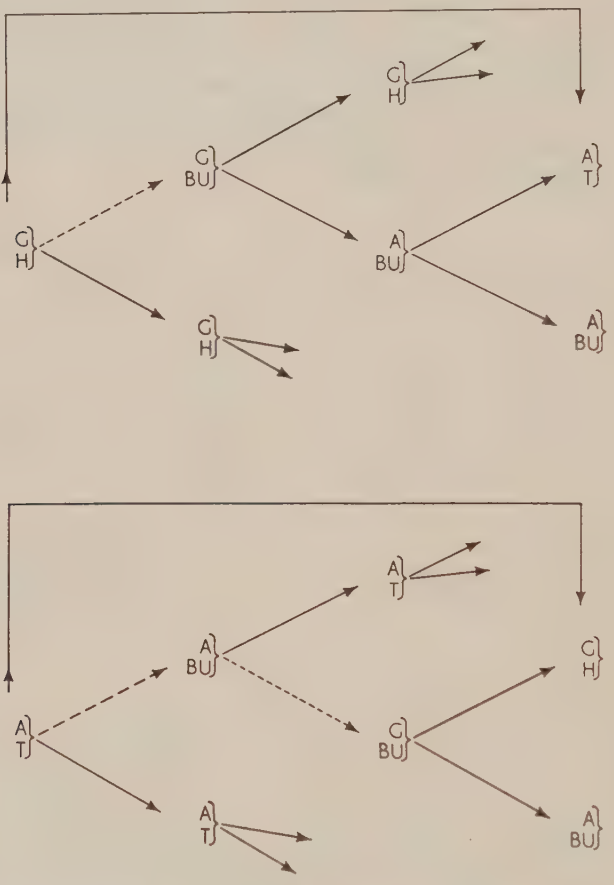


FIG. 6. *Illustration of the two types of pairing mistakes*, in the case of 5-bromouracil (BU). The normal DNA bases are indicated by H = 5-hydroxymethylcytosine, G = guanine, T = thymine, A = adenine.

(a) *Mistake in incorporation.* During DNA replication BU attaches " by mistake " to one of the G-bases, thereby initiating the change of an H—G into a T—A pair.

(b) *Mistake in replication.* During DNA replication BU attaches to adenine, with a frequency proportional to its incorporation into DNA. In one of the subsequent replications the incorporated BU may by mistake become attached to G instead of A, and thereby initiate the change of a T—A into a H—G pair. Under conditions of little BU incorporation two rare events are necessary, namely incorporation and mistake pairing.

Once a mistake in base pairing has occurred, the further development of the change is independent of the presence or absence of the base analogue in the medium. The

change of a nucleotide pair will produce an observable mutation if it causes the formation of proteins with altered function (e.g., a reactivated or inactivated enzyme).

This scheme has also been proposed by Meselson & Stahl (personal communication). It predicts that such a highly mutagenic base analogue can induce the "transition" of either of the two types of nucleotide pairs, adenine-thymine into guanine-HMC and *vice versa*. The relative frequency of transitions in these two directions cannot be predicted, at present, since it depends on several unknown factors. Experimentally, however, it may be possible to enhance or suppress one direction without altering the other. In an attempt to do this we have induced mutations under different growth conditions, e.g. adding together with BD four times as much thymidine, which should depress BU incorporation. So far essentially the same mutability spectrum has been observed under such altered conditions.

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Polynucleotides II : The X-Ray Diffraction Patterns of Solutions of the Randomly Coiled and Helical Forms of Polyriboadenylic Acid †

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It has been possible to obtain wide-angle X-ray diffraction photographs of dilute solutions of the acid form of polyriboadenylic acid in the form of sharp powder diagrams. The spacings so measured indicate a marked degree of similarity between the structure of the polymer in solution and in the solid state as a fibre. Consequently, the fibre can be identified with the acid form of the polymer. These observations are considered in terms of the two proposed models of the helical form of polyriboadenylic acid. It is concluded that they are only compatible with the model which has hydrogen bonds between the adenine groups of opposite chains as major cohesive forces for chain interaction. The ability of dilute solutions of acid-stable polyriboadenylic acid (helical form) to give sharp diffraction patterns is taken as evidence that individual molecules behave as microcrystals.

1. Introduction

Hydrodynamic and other physico-chemical studies of solutions of polyriboadenylic acid (poly A) have shown that this polynucleotide can undergo reversibly a type of helix-coil transition (Fresco & Doty, 1957; Fresco & Klemperer, 1959). The hydrodynamic random coil form is stable at neutral pH, where it exists as a single-chain, partially hydrogen-bonded, flexible polyelectrolyte. On lowering the pH so that at least 65 % of the adenine groups have been titrated, the net negative charge is reduced sufficiently to permit the single chains to associate to form organized linear complexes of varying length. These are negatively birefringent, highly extended, slightly flexible particles with hydrodynamic properties similar to those of undenatured deoxyribonucleic acid (DNA) (Doty, McGill & Rice, 1958). The molecular properties of this form have been interpreted in terms of a double-stranded helical model with discontinuities where one strand ends and another begins (Fresco & Doty, 1957; Fresco & Klemperer, 1959).

The X-ray diffraction studies of Crick, Davies, Rich & Watson (Crick, 1957; Rich, 1957; Watson, 1957; Crick, Davies, Rich & Watson, unpublished) on the solid fibrous state of poly A have led these investigators to propose a double-stranded helical model of the molecule with the bases on the inside and the phosphate groups on the outside. The bases are stacked 3·8 Å apart, essentially perpendicular to the ribose-phosphate backbone of each chain. Hydrogen bonds between the adenine groups of

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‡ Part of this work was done while the author, as Lalor Foundation Fellow, was a guest of the Cavendish Laboratory.

opposite chains, as seen in Plate I(a), provide the main cohesive forces in this model. Although the complete details of this model are not yet available, it is clear that the diffraction pattern, with its paucity of meridional diffraction, is that of a helical molecule; and the density and unit cell measurements are only compatible with a two-chain model.

A second interpretation of the fibre diffraction pattern has been advanced more recently by Morgan & Bear (1958). In their model, also a two-stranded helix, the chains are held together by pairs of hydrogen bonds between the furanose ring oxygen of one chain and the C-2 hydroxyl of the ribose of the other chain. The adenine groups are exposed on the periphery of the helix, and in any one helix are in Van der Waals contact in sets of two. The helices, in turn, are closely and regularly packed to form an ordered aggregate by means of interhelical hydrogen bonds between the peripherally-arranged adenine residues.

The present report deals with an investigation of the wide-angle diffraction of X-rays by the two forms of polyriboadenylic acid in solution. It appeared quite likely that the configuration of the acid-stable form in solution and that in the fibre were the same. A direct test of this was presumed possible, using the method of X-ray diffraction, since the very conditions necessary to observe Bragg reflections, as in a powder diagram, arising from intramolecular periodicities in dissolved macromolecules, seemed inherent in the acid form of poly A. If such reflections were observable, they should be absent at neutral pH where the periodic configuration was believed not to exist. Such a finding would provide firm support for the original interpretation of a pH-dependent helix-coil transition. Furthermore, similarity of configuration between the acid form of poly A in solution and the solid helical fibre would rule out the less likely structure of Morgan & Bear, for their model could not be expected to remain intact in solution. And, since the helical character of the fibre diagram is an unambiguous interpretation, direct evidence for the existence of a helix in solution would be provided. The results below demonstrate that these expectations were fully realized.

2. Experimental

The sample of polyriboadenylic acid used (sodium salt) had a molecular weight (as single chains) of 126,000 (Fresco & Doty, 1957). Solutions of the coil form were prepared by dissolving the polymer either in 0·15 M-sodium chloride + 0·015 M-sodium citrate at pH 7 or in water titrated to pH 7 or above. For the helical form, the polymer was dissolved in water at neutral pH and the solution so formed was titrated with dilute acid to the required pH. Homogeneity of the more viscous solutions was ensured by violently agitating them for several days.

Soft glass (Lindemann) capillary tubes, 0·3 to 0·7 mm in diameter, were filled with 1·5 to 10 % (wt/vol) solutions of poly A and sealed. They were mounted stationary with the tube axis vertical and perpendicular to the incident X-ray beam. A flat photographic plate was set normal to the axis of the X-ray beam 5·0 cm from the sample. Cu Kα radiation (λ = 1·54 Å) of very high intensity, filtered through nickel, was obtained from a rotating anode tube. Exposure times varied from 2 to 8 hr, depending on the tube focus and the concentration of poly A in solution. Control diffraction patterns were also obtained to ascertain the diffraction contributions of glass, water, and air.

3. Results

Typical diffraction patterns are shown in Plates I(b) and II(a). In Plate I(b) the photograph obtained from a solution of Poly A at pH 7 (randomly coiled form) is matched for comparison with a control pattern derived from the solvent in a capillary

tube. It is apparent that the randomly coiled form makes no detectable contribution to the diffraction pattern.† The diffuse rings which are present therefore represent the diffraction by water and the capillary tube glass, and indeed must be subtracted from the pattern given by the helical form.

The acid-solution pattern (helical form) shown in Plate II(a) contains, on the other hand, four readily observable diffraction rings at 3·8, 4·7, 5·8 and 10·3 Å. Partial orientation of the sample is evident in the photograph shown. This was achieved by drawing the viscous 10 % solution several millimeters up the fine capillary tube. When the solution was diluted, the inner rings eventually faded out, but at a concentration of 1·5 % the characteristic 3·8 Å spacing, which represents the translation of the nucleotide residues in the polynucleotide helix, remained readily discernible. The reversible appearance and disappearance of this spacing caused by appropriate change of pH of the same sample was observed repeatedly. There was no detectable change in any of the spacings as the pH was varied below the transition point, and to as low as 4·5.

4. Discussion

Plate II(b) is a fibre diagram of Poly A (by courtesy of A. Rich, 1957). It is striking that the very strong 3·75 Å meridional reflection has a spacing almost identical with the strongest ring (at 3·8 Å) in solution. Moreover, when solutions are partly oriented, the 3·8 Å ring is strongest on the meridian. In the fibre diagram the weak reflection just below that at 3·75 Å, and due to the Cu Kβ radiation for the same reflection, is absent in the solution diagram. Below this, there are two broad lines at 4·4 and 4·8 Å. These may be matched with the broad 4·7 Å ring in the solution diagram. Closer in, there is a broad, rather weak reflection at 10·2 Å, corresponding to the 10·3 Å ring in the solution pattern. Considering that we are comparing a fibre diagram with scattering from a solution, the degree of matching is excellent. There is only one significant discrepancy. The remaining strong reflection, which is probably on the first layer line, lies at 5·3 Å in the fibre, while in solution it appears at 5·8 Å. This difference could be due to a small alteration in the structure in solution. But a more likely explanation is that in solution the molecules are not regularly arranged and the spacing observed corresponds to the maximum of the continuous transform. In a fibre the molecules are in crystalline array and Bragg reflection from crystal planes takes place; so that the point at which the transform is sampled may be to one side of the maximum of the transform. As a result the spacing observed is different.

It is clear from this comparison that the helical fibre diagram of poly A can be identified with the acid-stable form of the polymer in solution. Evidently the structure is not a precarious one and persists in solution. This is consistent with the effects of proton addition on the solution properties of poly A which have been described previously (Fresco & Doty, 1957; Fresco & Klemperer, 1959).

Obviously, pH control of the medium is important for the preparation of negatively birefringent fibres of poly A. There is no indication in the published reports (Crick, 1957; Rich, 1957; Watson, 1957) that the pH was controlled in solutions from which fibres were drawn for X-ray analysis. This lack of pH control may explain why it was sometimes found impossible to prepare such crystalline fibres from some preparations

† Morgan & Blout (Abstracts of the 1958 meeting of the Biophysical Society, p. 34) have recently obtained an X-ray diffraction photograph of *stretched* films (only) of poly A cast from neutral (single chain) solutions of the polymer. The pattern indicates a regular configuration not helical in character.

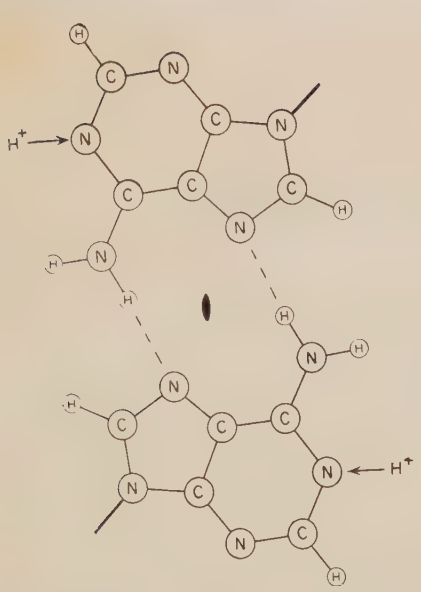

PLATE I(a). Hydrogen bonds between adenine residues of opposite chains in the structure of Crick, Davies, Rich & Watson. Modified after Rich (1957).

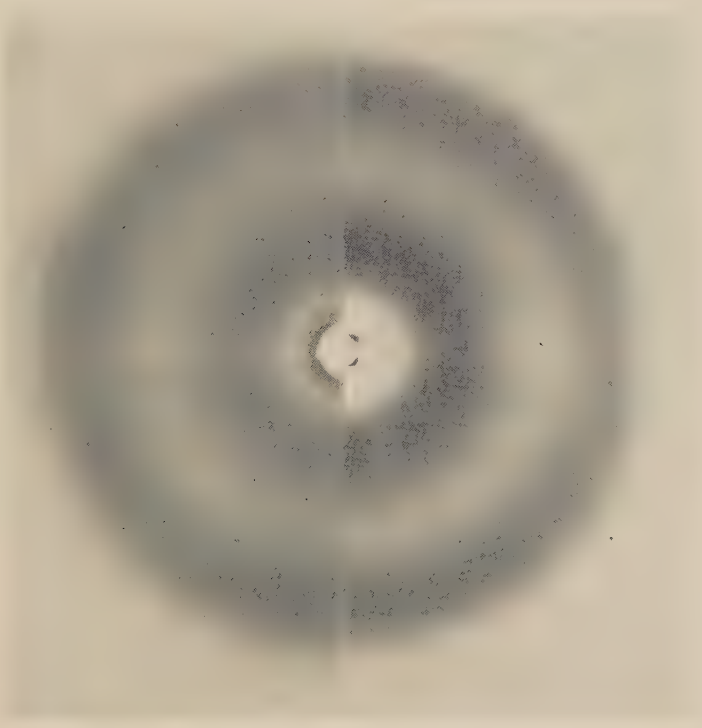

PLATE I(b). X-ray diffraction photograph of neutral 10 % solution of Poly A : right half. X-ray diffraction photograph of solvent : left half.

[*To face page* 108.

PLATE II(a). X-ray diffraction photograph of 10 % acid solution of Poly A.

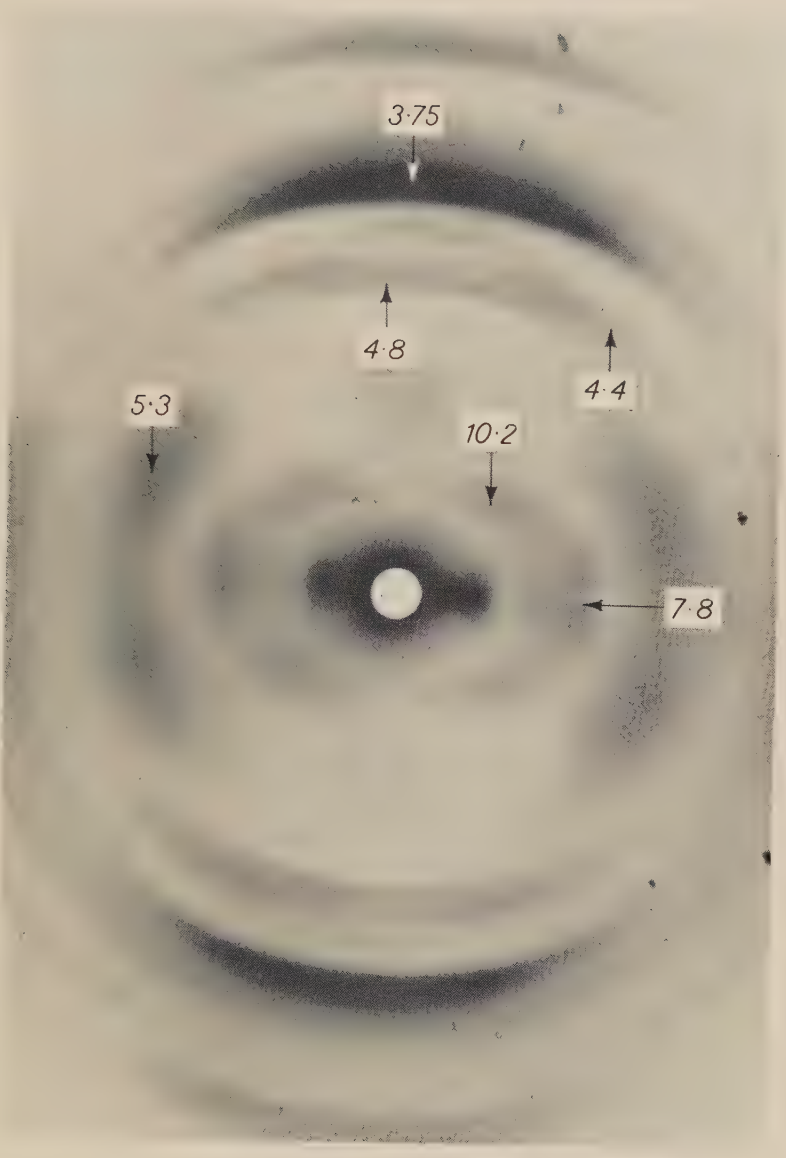

PLATE II(b). X-ray diffraction photograph of Poly A fibre (by courtesy of A. Rich, 1957). The numbers on the photograph indicate the spacings in Ångström units.

of poly A (Crick, 1957; Rich, 1957). However, if poly A fibres were drawn from solutions dialyzed against water, which is usually at about pH 5·5 owing to dissolved CO_2, it may be presumed that the polymers were in the helical form.

The identification of the fibre diffraction diagram with the acid form in solution implies that any structural model for the poly A helix be stable in solution. It is relevant in this connection to inquire into the nature of the diffracting units in acid solutions of poly A. These solutions give very sharp diffraction patterns, showing that the diffraction arises from a large number of highly ordered regularly repeating units. Several lines of evidence show that these units are located within single molecules which are not part of an *intermolecular* liquid-crystal lattice. The most dilute solutions studied (concn = 1·5 %) still sharply diffracted at the 3·8 Å spacing, and at this concentration the double-stranded poly A complexes are known to be hydrodynamically independent. Moreover, equatorial reflections, which would represent lateral arrangement of double-stranded polymer complexes (if they were part of a lattice) were not detected. The fibre diffraction photograph (see Plate II(b)) contains such an equatorial reflection at 7·8 Å, and it would be expected that, if it were likewise present in the solution scattering photograph, it would be discernible. Evidently, the high degree of order in acid solutions of poly A is of *intramolecular* origin, resulting from the one-dimensional periodicity of the adenine residues which are stacked essentially perpendicular to the long axis of the two-chained complexes. It is likely that this order in one dimension involves most if not all the dissolved polymer, for the diffracted beams are not broader than is observed with the fibre.

The model of Morgan & Bear (1958) depends for much of its stability on the integrity of an aggregate of two-stranded poly A helices. Clearly, this is at variance with the observation that the diffraction from acid solutions of poly A arises from the one-dimensional periodicity of molecularly dispersed helices. Apart from this, their model of the two-stranded helix fails in several respects. Since it is a structure with high charge density in its interior, it is not likely to be stable in solution; hydrogen bond interaction of the type proposed does not seem strong enough to overcome this factor. When protons are added to the adenine groups, there is no obvious way in which they can interact with the negatively charged phosphate groups of opposite chains. Although data presented elsewhere on solution properties (Fresco & Klemperer, 1959) strongly indicate base-pair interaction, this model leaves the marked spectral shift and loss of reactivity with formaldehyde at acid pH unexplained; i.e., the environment of the adenine groups in it are neither markedly restricted nor do they appear unavailable for chemical reaction.

In contrast, the structure of Crick, Davies, Rich & Watson (Crick, 1957; Rich, 1957; Watson, 1957; Crick, Davies, Rich & Watson, unpublished) offers several advantages. As shown in Plate I(a), there are two hydrogen bonds between adenine residues of opposite chains. In addition, two more hydrogen bonds (not shown) occur at the same level between the neutral oxygen atom of the phosphate group in one chain and the remaining hydrogen atom of the amino group of the adenine residue in the opposite chain. On examining the model with Dr. Crick it was found that the protons required for helix stability can be properly fitted on the adenine N-1 without markedly straining the structure.† In fact, this makes possible an electrostatic interaction with the negative oxygen atom on the phosphate group of the nucleotide

† The location of the proton on N-1 of adenine in poly A suggests a mechanism for the acid denaturation of DNA, since addition of a proton to this site, and by analogy to the N-1 of cytosine, would break the hydrogen bonds holding the two chains together. Relevant investigations with polycytidylic acid are in progress.

at the same level in the opposite chain. That this interaction makes a positive contribution to the stability of the helix is likely, since the helix is unstable even in neutral solution of high ionic strength, where the negative charges are shielded by the ionic atmosphere. In this connection, it should be noted that X-ray diffraction studies on adenine hydrochloride (Broomhead, 1948; Cochran, 1951) led to the conclusion that in this crystal there is adenine-adenine hydrogen-bonding of the type proposed for poly A, and that the proton is on N-1.

The analogy between the poly A model of Crick *et al.* and the two-chain helical structure of DNA (Crick & Watson, 1954) is apparent. Two significant differences stand out, however. One is that in the poly A structure there must be gaps or overlaps where individual polynucleotide chains end; even though these may be short they will weaken the structure. The other is that one of the hydrogen bonds in both DNA base pairs is an NH . . . O interaction, which is presumed to be stronger than the NH . . . N bonds in poly A (Pauling, 1940). Perhaps these factors are the reasons why the DNA helix is stable at neutral pH despite the high charge repulsion, whereas the poly A helix is only stable at a pH where its net charge is markedly reduced, and additional hydrogen bond and electrostatic interactions become possible.

The reinforcing character of wide angle diffraction of X-rays by dispersed helical poly A complexes in solution provides direct evidence for their occurrence as microcrystals. This diffraction observation appears to have been possible because of the high degree of periodicity and order in the side chain groups as well as in the polymer backbone. Apparently the presence, in the plane perpendicular to the chain axis, of only one type of dense diffracting group, the adenine heterocycle, has made our observation possible. Riley & Oster (1951) and Wilkins (1957) have observed X-ray diffraction by DNA gels. In this case, the strongest reflection, that arising from the stacked bases, fades out even at relatively high concentrations of DNA, suggesting that DNA is more irregular than poly A, due to a varied sequence of base pairs. Moreover, it is not apparent in the case of DNA that diffraction arising from independent molecules in true solution has been observed.

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The Preparation and Physical Chemical Properties of Ribonucleic Acid from Microsomal Particles

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Ribonucleic acid (RNA) prepared by phenol extraction from solutions of nearly homogeneous microsomal particles isolated from calf liver was found to consist of two nearly homogeneous components having sedimentation constants of 28 and 18 *S*. These two components occurred in the ratio of about 1 to 0·6 and could be assigned molecular weights of about 1,300,000 and 600,000 respectively. A molecule of each of these components would then account for the RNA content of a single microsomal particle which is found to have a molecular weight of 3,700,000.

These two RNA components appear to be constructed of subunits having molecular weights of about 120,000. Dissociation of the RNA components into these subunits is brought about by heating to 85° C. The RNA then has a sedimentation constant of about 8·2 *S* and exhibits a single, relatively narrow, sedimenting peak which resembled the RNA obtained previously by a detergent method followed by heating.

If the optical density at 285 mμ is followed during the heating cycle it is found to rise 27 % and return to its original value. On the basis of absorbance and optical rotation studies on polynucleotide helices reported elsewhere this is interpreted as indicating that about 40 % of the nucleotides are united through hydrogen bonds at room temperature and are released at higher temperatures. Upon cooling an equivalent number of hydrogen bonds re-form. The extent of reactivity with formaldehyde supports this interpretation. Similar studies on the microsomal particles themselves show an abrupt, reversible change that can be interpreted as arising from the melting out of base-paired regions that constitute about 30 % of the nucleotides. It is concluded that the phenol method, in contrast to the detergent method, preserves in the RNA that is isolated much of the secondary structure that existed in the microsomal particle.

1. Introduction

Particles 200 Å in diameter which consist of about equal amounts of ribonucleic acid and protein are present in the cytoplasm of bacteria, in many mammalian cells, and in rapidly growing plant cells (Schachman, Pardee & Stanier, 1952; Petermann & Hamilton, 1957; Tso, Bonner & Vinograd, 1956). In the liver these particles are attached to membranes which, upon physical disintegration of the cell, constitute the microsome fraction (Palade & Siekevitz, 1956). Accordingly, these attached nucleoprotein particles are commonly designated microsomal particles.

Because of the ability of these particles to incorporate amino acids rapidly *in vivo* (Littlefield, Keller, Gross & Zamecnik, 1955), and because of the apparent regularity and uniformity of the structural pattern of the microsomal particles and the presence in them of highly polymerized RNA, it is now widely believed that these particles function as templates for the assembly of amino-acids in the proper proportion and sequence for polymerization into specific proteins. In order to gain some insight into the workings of such an RNA template and to demonstrate that such templates exist, a knowledge of the physical organization of the template would seem to be

essential. We have undertaken a study of the ribonucleic acid in the microsomal particles of liver with this purpose in mind.

RNA prepared from liver microsomes by Grinnan & Mosher (1951, 1953), using guanidine hydrochloride to denature protein, had a sedimentation coefficient of 6·4. Their estimate of the molecular weight of the RNA was 150,000. More recently, Pain & Butler (1957) have reported a molecular weight of 30,000 for RNA from microsomes. They prepared RNA by acid precipitation of nucleoprotein, followed by heat treatment and digestion with chymotrypsin.

In our first efforts on this problem we prepared RNA from microsomal particles using detergent, followed by heating. The physical chemical properties of RNA obtained in this way are described in an earlier paper (Hall & Doty, 1958). In summary, the properties of this RNA in solution are those of a coiled single chain polymer. In solutions of moderate ionic strength, the hydrodynamic properties indicate a degree of coiling which is considerably tighter than one would anticipate for a randomly coiled polymeric electrolyte. Apparently this is a result of hydrogen bonding between groups of nucleotides located in different regions of the same RNA chain. The molecular weight of the heated detergent RNA is 110,000 $\pm$ 15,000 and the sedimentation coefficient is about 7 *S*. Both the sedimentation properties of this RNA and the very high viscosity of its solutions in pure water are remarkably similar to the observations of Grinnan & Mosher (1953) on RNA prepared with guanidine hydrochloride. It appears likely that, in their preparation, the hydrogen-bond breaking action of guanidine hydrochloride accomplished what heating RNA to 83°C did in our experiments—the destruction of interchain hydrogen bonds, resulting in the release of individual polynucleotide strands.

The apparent molecular weight of the detergent RNA before heat treatment was two to four times that of the heated material. The unheated RNA appeared to be in solution as a somewhat unstable aggregate which exhibited a gradual fall in intrinsic viscosity and sedimentation constant. After heating these quantities were constant. The sedimentation boundaries indicated a far greater polydispersity of size in the unheated RNA than in the heated RNA, for which the boundaries were sharp and single-peaked.

In our previous investigation it was not possible to determine whether the aggregation of unheated detergent RNA was a vestige of the structure of RNA in the microsomal particles or whether the aggregates were formed during the preparation. This question has been answered in the present work by preparing the RNA in a different manner: extraction with aqueous phenol at low temperature. RNA prepared in this way resembles the detergent RNA in that it is a high molecular weight aggregate, unstable at 25°C. It differs markedly, however, in that the sedimentation boundaries consist initially of two clearly resolved components, within each of which the range of sedimentation coefficients is quite narrow. The uniformity of these RNA components suggests very strongly that the arrangement of RNA chains in them is a manifestation of the organization of the RNA in the microsomal particles.

2. Experimental

(a) *Preparation of microsomal particles*

A fresh calf liver, weighing about 0·6 kg, was cut into strips 1 cm thick and frozen in dry ice 15 to 30 min after slaughter. For each preparation of microsomal particles 200 g of the frozen liver, previously stored under dry ice in a Dewar jug, were allowed to thaw at 5°C. The liver was chopped very finely with a meat cleaver, in order that fibrous material would not clog the blender. The chopped liver was homogenized in 360 ml. of 0·25

M-sucrose solution. This was accomplished by blending the liver in two lots in an Osterizer mixer. The mixer was run in the cold at half-speed intermittently for 3 to 4 min to produce a homogeneous suspension. This was centrifuged twice for 30 min at 1500 *g* in the cold. The supernatant solution, containing the microsomes, glycogen, and soluble liver proteins, was removed by pipette after each centrifugation. After the second centrifugation, the supernatant was brought to pH 8·0 by dropwise addition of 0·5 N-NH_4OH. Forty ml. of a 5 % solution of sodium deoxycholate in 0·01 M-tris buffer, pH 8·0, were added slowly with stirring; stirring was continued for 15 min at 5°C. Microsomal particles were separated from the deoxycholate suspension by centrifugation for 10 hr at 60,000 *g*. The supernatant solution was poured off and the sediment suspended in 0·5 % sodium deoxycholate, 0·01 M-tris, pH 8·0, with the aid of a teflon-glass pestle homogenizer. This suspension was centrifuged for 20 min at 15,000 *g*; the clear supernatant solution was removed by pipette and then centrifuged for 3 hr at 100,000 *g*. The supernate was removed from the microsomal particle pellets and these were suspended in 0·02 M-KH_2PO_4-K_2HPO_4, pH 7·0, using a pestle homogenizer. This was centrifuged for 15 min at 10,000 *g*. The nearly colorless supernatant solution was decanted and centrifuged for 2 hr at 100,000 *g*. The pellets were again suspended in 0·02 M-phosphate buffer and the low and high speed centrifugation steps repeated. Purification of the microsomal particles was not routinely carried beyond this point. The pellets of microsomal particles were suspended in 0·02 M-phosphate buffer if RNA was to be prepared directly from them. If the microsomal particles were to be preserved for a substantial period of time, a solvent containing less salt and a small amount of $MgCl_2$ was employed as, for example, 0·025 M-$NaHCO_3$, 0·005 M-$MgCl_2$ or 0·001 M-phosphate buffer + 0·0005 M-$MgCl_2$. In either case, the final solution of microsomal particles was centrifuged briefly (10 min at 10,000 *g*) to remove large aggregates of microsomal particles. A yield of 400 to 500 mg of purified microsomal particles was obtained from 200 g of calf liver.

(b) *Preparation of RNA*

The method of phenol extraction of protein from an aqueous nucleoprotein solution, first used by Gierer & Schramm (1956) and later by Colter & Brown (1956), was used with one modification. Treatment of the microsomal particles for 5 min at 20°C with 0·1 % sodium lauryl sulfate, before the phenol extraction, brought about a more rapid and complete separation of protein from RNA than was obtained with phenol alone.

A solution of microsomal particles in 0·02 M-phosphate buffer, pH 7, at a concentration of nucleoprotein between 0·1 and 1 g/100 ml. was used for the preparation of RNA. To 30 ml. of this solution at 20°C was added 0·12 ml. of a solution containing 25 g sodium lauryl sulfate per 100 ml. This was stirred for 5 min and then placed in an ice bath. All subsequent steps in the preparation were carried out in a cold room (5°C). The solution of microsomal particles was shaken for 8 min with 30 ml. of water-saturated phenol † in a long-necked flask on a wrist-action shaker. The phenol and aqueous phases were separated by centrifugation for 2 min at 5000 rev/min in a Servall angle centrifuge. Most of the protein was removed in this step. The RNA-containing aqueous layer was separated from the opaque protein-phenol gel by pipetting. The RNA solution was shaken two additional times for 2 min with 10 ml. phenol-water, followed in each case by centrifugation and removal of the aqueous layer by pipette. Extraction of the solution three times with 100 ml. ether removed most of the phenol. The ether was vaporized by bubbling nitrogen through the solution. Small amounts of phenol or other low molecular weight impurities left behind were separated by dialysis for 3 to 5 hr against 0·02 M-KH_2PO_4-K_2HPO_4, pH 7·0. Protein contamination of the RNA was undetectable by the biuret test: this would limit the amount that could be present to less than 0·5 %. For purposes of brevity, ribonucleic acid prepared by this method will be designated simply as RNA.

(c) *Techniques and apparatus*

All viscosity measurements were made in Ostwald-Fenske viscometers having flow times of approximately 200 sec for water. Dust and fibers were removed from the solutions by centrifugation, to insure reproducible flow times.

† A short time before use the phenol was redistilled. This was necessary to remove strong acid impurities present in the phenol.

Sedimentation velocity runs were done in a Spinco Model E Ultracentrifuge using the ultraviolet absorption optical system. The sedimentation boundaries were recorded photographically on X-ray film. Graphical representation of RNA concentration as a function of position in the cell was obtained from the films using a Spinco Analytrol densitometer. All sedimentation measurements were made at 4 $\pm$ 1°C. The sedimentation coefficients were corrected for solvent density and viscosity to 20°C.

A Beckman Model DU spectrophotometer was used for routine concentration determination and for measurements of ultraviolet spectra and of the variation of ultraviolet absorption with temperature. For the latter experiments, the temperature of the solution was controlled by circulating water from a thermostat through coils in the walls of the cell compartment.

The concentration of RNA solutions was determined by the optical density of the solution at 260 mμ. A value of 190 was used for the extinction of solution containing 1 g RNA per 100 ml. in 0·02 M-phosphate buffer (Kay & Dounce, 1953) at 25°C. (The extinction coefficient of liver microsomal RNA may actually be somewhat higher than this. From the base ratios (Crosbie, Smellie & Davidson, 1953), the molar absorptivities of the 3′-nucleotides (Beaver, Holiday & Johnson, 1955) and the 39 % hyperchromic effect we observe on alkaline hydrolysis of this RNA, a value of 214 is calculated.)

An approximate value of 0·55 for the partial specific volume of RNA has been used throughout for calculations of molecular weight from the sedimentation coefficient.

3. The Properties of RNA in Solution

(a) *Viscosity, sedimentation properties and molecular weight of RNA*

Each RNA preparation was characterized by sedimentation-velocity analysis immediately after the dialysis step was completed. In Fig. 1 are shown the sedimentation boundaries for four preparations. Although the relative amounts of the

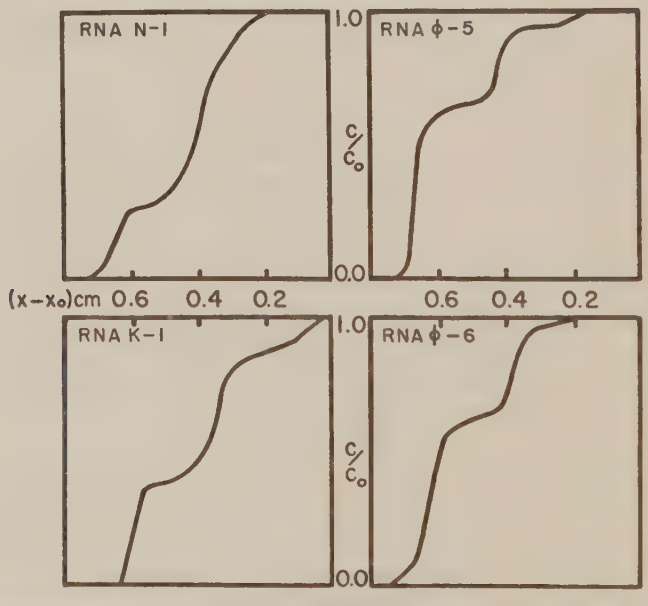

Fig. 1. Sedimentation boundaries of typical RNA preparations. The boundaries are shown after approximately 20 minutes at 59,780 rev/min.
Solvent: 0·02 M-KH_2PO_4–K_2HPO_4 (equimolar).
RNA concentration: about 50 μg/ml.

two components were somewhat variable, the average sedimentation rate for each component was the same within experimental error in all preparations. The variation shown by these four preparations in the sharpness of the boundary and in the amounts

of the two components is representative of all preparations studied. The sedimentation properties of different RNA preparations were irreproducible to the extent that a pattern similar to any one of these might be observed for a given RNA preparation. In general, however, less diffuse boundaries and larger amounts of the 28 *S* component seemed to result when the elapsed time during the isolation of microsomal particles was shortest. All experiments other than these sedimentation studies were carried out on the two preparations having the highest proportion of 28 *S* RNA; both had a ratio of 28 *S* to 18 *S* components of 1·65.

The reduced specific viscosity of RNA was measured in 0·02 M- and 0·05 M-K_2HPO_4-KH_2PO_4 (1 : 1). At 25°C, the viscosity decreased with time; measurement of η_{sp}/c at a series of concentrations was therefore impracticable. The initial values of the reduced specific viscosity in the two solvents are given in Table 1.

TABLE 1

Reduced specific viscosity of RNA

RNA concentration	η_{sp}/c	solvent
0·40 g/100 ml.	0·44	0·02 M
0·19	0·38	0·05 M

The reduced specific viscosity in dilute salt solutions of heat-treated detergent RNA obtained from microsomal particles is greater than the intrinsic viscosity by an amount which is proportional to RNA concentration, in accord with Huggins' equation (1942):

$$\frac{\eta_{sp}}{c} = [\eta] + k'[\eta]^2 c$$

For the detergent RNA k' has the value 0·4. Assuming this value to be applicable also to the data of Table 1, the calculated intrinsic viscosity of RNA is 0·37 in 0·05 M-KH_2PO_4-K_2HPO_4 and 0·41 in 0·02 M buffer. Provided the 18 *S* and 28 *S* species of RNA are homologous with regard to frictional properties, then the intrinsic viscosity of 0·41 for the mixture would not differ greatly from the intrinsic viscosity of the 28 *S* component. The sedimentation-viscosity molecular weight calculated from this, being relatively insensitive to the intrinsic viscosity, would be only slightly in error. On this basis a molecular weight of 1,300,000 is obtained from the Scheraga-Mandelkern equation (1953):

$$\frac{s^\circ [\eta]^{1/3}}{M^{2/3}} = \beta \frac{(1 - \bar{V}\rho)}{\eta_\circ N}$$

assuming the value of β to be $2{\cdot}35 \times 10^6$. This value of β was obtained experimentally for RNA of 105,000 molecular weight and is in the range that is generally accepted for randomly coiled polymers. However, the RNA presently under consideration may have quite another effective hydrodynamic shape, in which case the appropriate value of β might differ somewhat from $2{\cdot}35 \times 10^6$. The uncertainty in the molecular weight of the 28 *S* RNA arising from this may be at least 300,000.†

† A value of β as small as $2{\cdot}1 \times 10^6$ (the value for spheres) or as large as $2{\cdot}6 \times 10^6$ would seem to be quite as likely as that used, $2{\cdot}35 \times 10^6$. In the former case the calculated molecular weight is 1,600,000 and in the latter 1,100,000; from these the minimum uncertainty in the molecular weight of RNA is estimated to be 300,000.

The molecular weight of the 18 *S* RNA component is a more elusive quantity than that of the 28 *S* component. Judging from the symmetric spreading of the sedimentation boundaries of the two components, the 18 *S* component would appear to have a larger diffusion coefficient than the 28 *S*. Since the sedimentation coefficient is smaller as well, the 18 *S* component apparently has the lower molecular weight of the two. The relatively high sedimentation coefficients and low intrinsic viscosity are indicative of a compact or tightly coiled configuration of the RNA chains. Provided the two components are homologous in this respect, the molecular weight of the 18 *S* component would be approximately one-half that of the 28 *S* component.† If the latter were 1,300,000, the 18 *S* component would have a molecular weight of approximately 600,000.

These results are remarkably similar to those obtained by Timasheff, Colter, Brown & Davies (1958) in studies of the high molecular weight fraction of Ehrlich Ascites tumor RNA. They found the molecular weight (weight average) of Ehrlich Ascites RNA to be 1,200,000 and the intrinsic viscosity 0·4. Two components were observed in the ultracentrifuge having sedimentation coefficients of 32 *S* and 15 *S* in a ratio of 1·5 : 1. Because of the close correspondence in the properties of these two types of RNA, it appears quite likely that the RNA extracted from Ehrlich Ascites cells was present intracellularly in microsomal particles.

(b) *Hydrogen-bonding and changes in the ultraviolet absorption of RNA*

The question of the type of secondary structure present in the high molecular weight RNA is one of the first to occur. In view of the importance of hydrogen bonding between purine-pyrimidine pairs in other polynucleotides, e.g., deoxyribonucleic acid, polyadenylic acid + polyuridylic acid, and polyinosinic acid + polycytidylic acid, we attempted to estimate the extent and importance of this type of hydrogen bonding in microsomal particle RNA.

One method we have used to study hydrogen-bonding in RNA is based upon the hyperchromic effect produced by increasing the temperature of an RNA solution and the interpretation of this in terms of hydrogen-bonding. A similar effect of temperature upon the ultraviolet absorption of DNA was noted by Thomas (1954). Cox & Peacocke (1954) found that the irreversible increase in ultraviolet absorption was a linear function of the extent of denaturation of the DNA, as measured by the change in the number of amino groups titrated at a given pH. This effect has been placed on a quantitative basis by a concurrent investigation in this Laboratory (Doty, Boedtker, Fresco, Hall & Haselkorn, 1959) in which the changes in optical density at 260 mμ and optical rotation accompanying helix-coil transitions in polynucleotides were used to calibrate a scale of percentage of base pairs in helical configuration. The temperature dependence of optical density and optical rotation in RNA was then found to be consistent with rupture of the same fraction of hydrogen-bonded base pairs.

RNA isolated from liver microsomal particles with detergent exhibited a considerable hyperchromic effect upon heating; the optical density at 258 mμ rose by 27 %

† In the empirical equation $s^\circ = KM^\alpha$, describing the dependence of sedimentation coefficient upon molecular weight, the exponent α approaches 0·5 for very tightly coiled polymers. For heat-treated detergent RNA, α was 0·49. Provided this exponent is applicable also in the case of tightly coiled 18 *S* RNA and 28 *S* RNA, the ratio of molecular weights would be:

$$\frac{M_{18\,S}}{M_{28\,S}} = (18/28)^{1/0.49} = 0.43$$

between 25°C and 85°C in 0·01 M-phosphate buffer (Hall & Doty, 1958). There was, however, no irreversible change; that is, the optical density measured at 25°C was not affected by heating the solution to 85°C. The set of hydrogen bonds which is broken by heating is apparently not a unique one, as it is in the case of DNA. The 27 % increase in the optical density of RNA between 25°C and 85°C is more than two-thirds the total optical density change occurring on alkaline hydrolysis of RNA. (A 39 % increase in the optical density at 258 mμ, measured in 0·05 M-K_2HPO_4-KH_2PO_4 at 25°C, was observed when RNA was hydrolyzed for 12 hr at 40°C in 1 N-NaOH.) As expected, the optical density of the mixed mononucleotides was independent of temperature, the range of measurement being 25°C to 70°C.

The effect of increasing temperature upon phenol-RNA is quite the same as with the detergent preparations. In Fig. 2 is shown the optical density at 258 mμ as a function of temperature for RNA in 0·02 M-phosphate buffer, pH 7·0, and for detergent

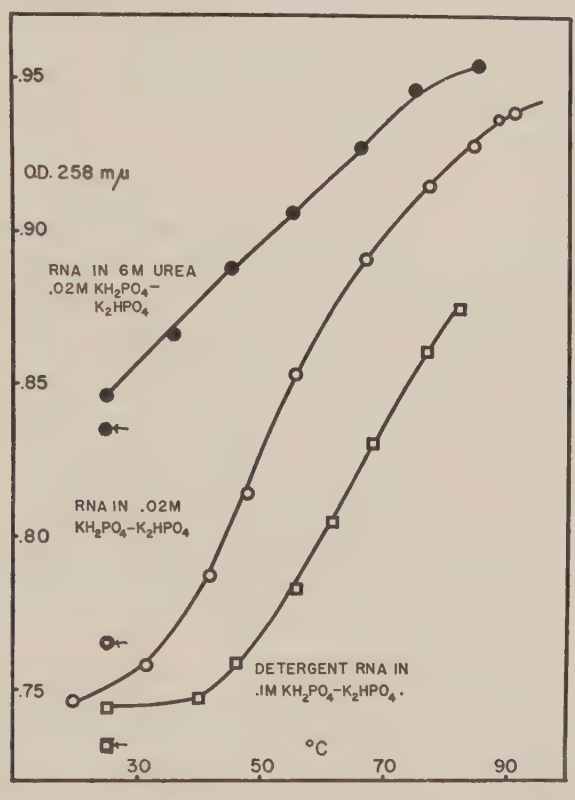

FIG. 2. The temperature dependence of the optical density of ribonucleic acid. Circles refer to phenol-prepared RNA with (●) and without (○) 6 M-urea present in 0·02 M-phosphate buffer. The squares □ refer to detergent-prepared RNA in 0·1 M-phosphate buffer. The symbols with an arrow attached indicate the optical density after cooling the heated solutions. (Correction was made for thermal expansion.)

RNA in 0·1 M-phosphate. Also shown is the optical density of RNA in 6 M-urea—0·02 M-phosphate. The urea appears to decrease the extent of hydrogen bonding, particularly at low temperature. Note the absence, in all three cases, of any significant difference in the optical density measured at 25°C before and after heating. At each temperature, an equilibrium value of the optical density is attained rapidly.

(c) *The reaction of RNA with formaldehyde*

The reaction between formaldehyde and the amino groups of cytosine, adenine and guanine in RNA has been studied extensively by Fraenkel-Conrat (1954) and Staehelin (1958). The reaction may conveniently be followed spectrophotometrically, for it is accompanied by a large increase in optical density in the region 255–284 mμ as well as a shift of the absorption maximum to higher wavelength. Because the reaction of formaldehyde with free amino groups is rapid whereas hydrogen-bonded groups react slowly, this reaction is quite useful for the study of hydrogen-bonding in RNA. The results of studies we have made of the reaction of formaldehyde with microsomal particle RNA are entirely consistent with the conclusions of the preceding section regarding hydrogen-bonding in RNA.

In the first experiment, the results of which are shown in Fig. 3, the reactivity with formaldehyde of heated RNA (15 min at 85°C) was compared with that of unheated RNA. Heated and unheated RNA were indistinguishable in the rate and extent of

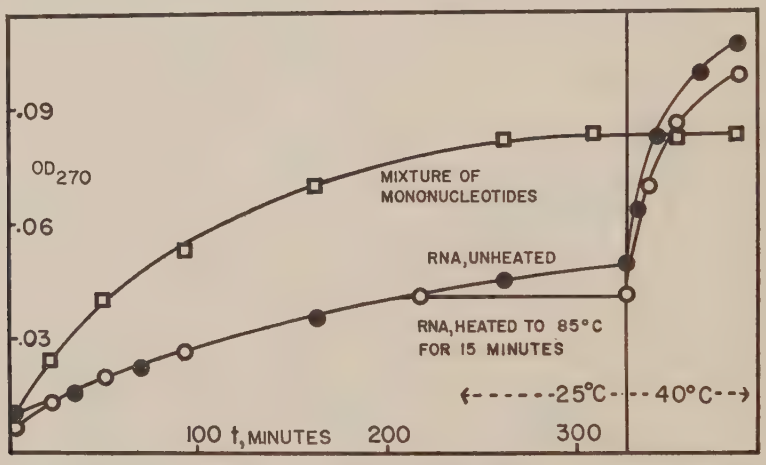

Fig. 3. The reactivity of RNA and the corresponding mononucleotides with formaldehyde at 25°C and 40°C. The optical density at 270 mμ is shown as a function of time. After 325 minutes the temperature was changed from 25°C to 40°C.

their reaction with formaldehyde, both at 25°C and 40°C. The rate of reaction at 25°C was one-fourth that of the mixed mononucleotides, and only a fraction of the amino groups reacted over a long period of time. At 40°C additional amino groups became available for reaction with formaldehyde, presumably as a result of decreased hydrogen bonding. The formaldehyde concentration was 0·5 %.

In another experiment we studied the conditions necessary for complete reaction of the amino groups of RNA with formaldehyde. The change in the ultraviolet spectrum of RNA was found to be complete after 10 hr at 80°C, but not after 6 hr at 60°C. Since at 80°C all the amino groups appear to have reacted with formaldehyde and can no longer form hydrogen bonds, no hyperchromic effect should occur on cooling. We found a decrease of only 2 % in the optical density at the absorption maximum on cooling from 80°C to 25°C, as compared to the 27 % decrease in the case of RNA not reacted with formaldehyde.

This reaction with formaldehyde was carried out in 0·001 M-phosphate buffer. At this low ionic strength, hydrogen bonding in RNA is diminished even at 25°C, as

evidenced by the more extended molecular configuration and the altered temperature dependence of the ultraviolet absorption. As one might expect, the initial reaction with formaldehyde at 25°C proceeded twice as fast in 0·001 M-phosphate as it did in 0·05 M-phosphate.

(d) *The extent of hydrogen-bonding in RNA*

Using the data for the reaction of formaldehyde with RNA in 0·05 M-phosphate, it is possible to estimate roughly the degree of hydrogen-bonding in RNA under these conditions. Provided the reaction is first order with respect to free amino groups, the ratio of the initial rates of increase in optical density at 270 mμ for RNA and for the mononucleotides would equal the ratio of the concentrations of reactive amino groups. The rate of increase in optical density at 270 mμ for RNA was 40 % of that of the mononucleotides; therefore, if all the amino groups of the mononucleotides are reactive, then 60 % of the amino groups of RNA are unavailable for reaction with formaldehyde.

If it is assumed that all the free amino groups of RNA reacted in 5 hr at 25°C and that complete reaction occurred at 40°C (12 hr), then the fraction of amino groups not hydrogen-bonded at 25°C would equal the fraction of the total increase in optical density at 270 mμ which occurred at 25°C. This fraction was equal to 35 %, which corresponds to 65 % hydrogen-bonding.

A more reliable estimate of hydrogen-bonding in the RNA is available from the combined ultraviolet absorption and optical rotation study involving synthetic polynucleotides (Doty *et al.*, 1959). From this it was concluded that 40 % of the nucleotides are hydrogen-bonded in liver microsomal RNA at 25°C. Although this pairing can be broken by heating and reformed on cooling this large amount of base pairing must be considered as a characteristic feature of RNA under the conditions where it is generally studied, i.e., room temperature in the presence of some salt.

With such a high density of hydrogen bonds it is natural to ask if the RNA components obtained from the microsomal particles might not consist of a number of RNA subunits, that is individual molecular chains, held together by some of these hydrogen bonds. With this in mind the apparent molecular weight was followed as the hydrogen bonds were broken by raising the temperature.

(e) *The thermal dissociation of RNA*

The effects of heating upon the molecular weight and configuration of RNA were followed by measurement of the irreversible change in viscosity produced by exposure to successively higher temperatures for a fixed period of time. Figure 4 shows the results of two such experiments. The reduced viscosity was measured after exposure of the solution for ½ hr to each of the temperatures for which a point is shown. In both cases, the viscosity began to fall at quite a low temperature, decreasing most rapidly in the range from 40° to 80°C. In 0·05 M-phosphate, the reduced viscosity approached a lower limit of 0·17 at high temperature. In 0·01 M-phosphate, using detergent RNA from the same source, molecular weight (measured by light scattering), reduced viscosity, and sedimentation coefficient all fell to a lower limiting value after heating at 83°C. Although no such lower limit is evident in the curve of viscosity of RNA *versus* temperature of heating in 0·02 M-phosphate (Fig. 4), this may be attributed to the small number of points recorded (only one above 65°C) in the temperature range in which the dissociation of RNA nears completion.

In 0·02 M-phosphate the reduced viscosity fell to 0·22 as a lower limiting value. A sedimentation diagram with ultraviolet optics was obtained for RNA subjected to a similar heat treatment. No longer were two definite components present; the boundary was smooth and continuous. The mean sedimentation coefficient of RNA heated to 85°C for 15 min in 0·02 M-phosphate was 8·2 *S*.† This, together with an intrinsic viscosity of 0·22, would place the molecular weight of the heated RNA at 120,000. This is in quite close agreement with the molecular weight obtained for heated RNA prepared with detergent (Hall & Doty, 1958). Furthermore, the values obtained for the sedimentation coefficient and intrinsic viscosity are precisely those which the heated detergent RNA would display in this solvent. Thus, it appears likely that the RNA molecules which result when this RNA is heated are single chain coils as are the molecules of heated detergent RNA. The attainment of an approximate limiting size of RNA by different methods suggests that the 18 and 28 *S* components of RNA are constructed of subunits. However, the possibility remains that these subunits are artifacts caused by hidden scissions being induced by transient ribonuclease action during the breakdown of the microsomal particle.

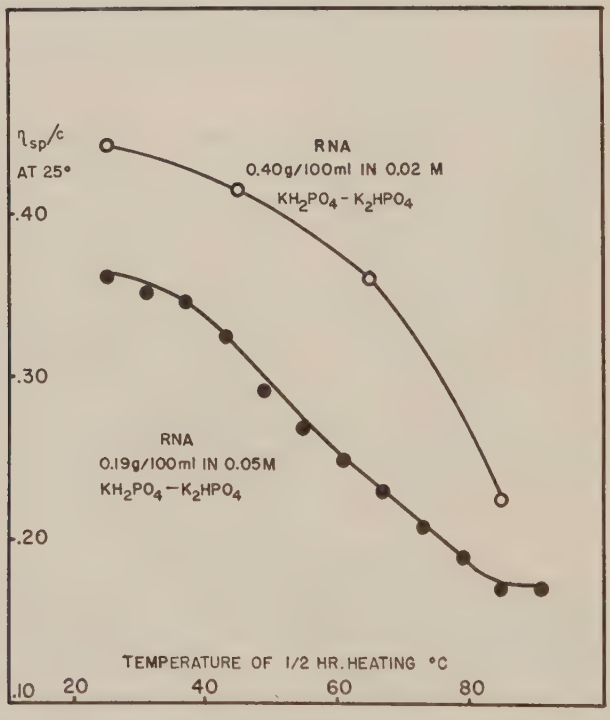

Fig. 4. The variation of the viscosity of RNA solutions with thermal treatment.

(f) *The interpretation of the temperature dependence of the hydrogen-bonding*

Two quite different interpretations of the temperature dependence of hydrogen bonding can be advanced. Although we prefer one to the other, both will be described briefly.

† In a more detailed study of the effects of heat upon the sedimentation behavior of RNA, the bimodal nature of the sedimentating boundary was destroyed by heating for 5 minutes at 60°C. This treatment reduced the average $S_{20,w}$ to 11 *S*. After heating for 20 minutes at 80°C, the average sedimentation coefficient was 9·3, and the sedimentation distribution was sharpened further. Heating to 90° for 5 minutes reduced the average *S* to 8·5, but did not bring about any additional narrowing of the distribution. The solvent in this case was 0·05 M-KCl and 10^{-4} M-Tris (pH 7).

One interpretation is based on the view that the groups of hydrogen-bonds that form intramolecular and intermolecular linkages are equivalent and that the curve of optical density *versus* temperature reflects the partitioning of these linkages between those that are closed and those that are open. Thus the dissociation of these linkages would be governed by a single equilibrium constant and the temperature dependence of the phenomenon would be related to the magnitude of the enthalpy change accompanying the break of a linkage. This hypothesis can be tested, assuming that the optical density at low and high temperatures corresponds to 0 % and 100 % dissociation of the linkages and that at intermediate temperatures it is proportional to the fraction of broken linkages. On this basis the test would consist in plotting the logarithm of the equilibrium constant against reciprocal absolute temperature. The equilibrium constant, on the basis of the above assumption, is $(OD - OD_T)/(OD_T - OD_0)$, where OD is optical density, no subscript refers to the high temperature limit, T refers to the temperature in question, and O refers to the low temperature limit. This plot is shown in Fig. 5 and it is seen to be linear indeed. The enthalpy

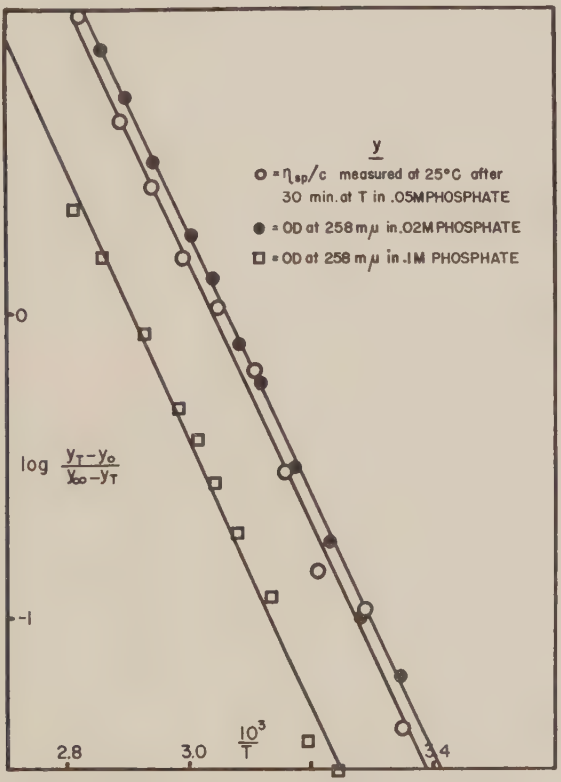
y
○ = η_{sp}/c measured at 25°C after 30 min. at T in .05M PHOSPHATE
● = OD at 258 mμ in .02M PHOSPHATE
□ = OD at 258 mμ in .1M PHOSPHATE
0
-1
log $\frac{y_T - y_0}{y_\infty - y_T}$
2.8
3.0
3.4
$\frac{10^3}{T}$

FIG. 5. A van't Hoff plot of the temperature-dependence of the viscosity and ultraviolet absorption of microsomal RNA.

change, derived from the slope, is 20 kcal/mole of linkages, and the entropy changes are 56 and 59 entropy units in the two different ionic strengths. Since the heat of dissociation of a nucleotide pair probably lies within the range of 3 to 8 kcal this would suggest that the linkages are made of several adjacent nucleotide pairs. Thus far it is seen that this interpretation is consistent with the data and the size of the linkage region appears reasonable. However, this view requires that

the equilibrium be a dynamic one with linkages continuously breaking and reforming. The limited reactivity of formaldehyde is strong evidence that this is not the case.

The other interpretation lies in the discovery of cooperative helix-coil transitions in polypeptides, polynucleotides, and deoxyribosenucleic acid in the last couple of years. These studies have shown that helical structure supported by hydrogen bonds can undergo transitions to disordered states in such a way that the temperature profile has the same character as that found for RNA in this work. While in many cases these transitions are much sharper than that observed here, there are cases where it is similarly broad and can be fitted by a van't-Hoff plot in the same way. In addition, we have in the case of RNA other reasons to expect such a transition to be broad; the shortness of the helices, imperfections within the helices (inclusion of occasional mismatched base pairs) and variations in the heats of dissociations of various nucleotide pairs within the helical regions. The exploration of this interpretation is considerably more difficult than the former one and it has been undertaken with the inclusion of evidence from other systems elsewhere (Doty *et al.*, 1959). For the present it is sufficient to say that substantial support can be obtained for this view: that the hydrogen-bonding potential of RNA chains is so great that clusters of nucleotide pairs develop spontaneously at room temperature. The higher the ionic strength the more temperature-resistant these are. In RNA these helical regions occasionally unite different chains thereby forming a network. As the temperature of the solution of these networks is raised the weaker linkages melt out first, releasing the subunits that were held only by these linkages. The progressive melting out of the stronger linkages occurs with subsequent release of subunits until the strongest linkage is gone and the thermal dissociation is complete. When the temperature is lowered at the high dilution employed in this work (0·005 g/dl.) the helical regions of nucleotide pairs reform intramolecularly.

Although these two interpretations are quite incompatible in principle they both present the same anatomical view of RNA in solution at room temperature: a highly coiled structure in which about 40 % of the nucleotides cluster together in short stretches in hydrogen-bonded arrays that are probably helical. The difference lies mainly in whether these arrays have identical bonding strengths or not. In both interpretations the gradual disintegration of the phenol-RNA components with rising temperature is expected as the subunits are shed, provided these subunits are held in the network by only a small fraction of the linkages.†

(g) *Conclusions*

It appears that the following conclusions can be drawn concerning the RNA of calf liver microsomal particles.

(i) The RNA isolated by the phenol method consists of two components having particle weights of about 1,300,000 and 600,000. These occur in the weight ratio of about 1 : 0·6. Since a particle of each component taken together corresponds closely to the RNA content of a microsomal particle it is likely that the RNA in the microsomal particle consists of one of each of these components.

(ii) Upon heating to 85°C the two components break up to yield a single component of 120,000 molecular weight. This component is considered to consist of individual

† Otherwise the thermal dissociation would be concentrated at the high temperature end where the last linkages are being broken. That the dissociation of the subunits occurs throughout the region of temperature dependence of optical density is seen in Fig. 5, where a plot of the viscosity is made in the same way as the optical density, and a linear plot results.

RNA chains. These subunits are very similar to the RNA obtained by heating RNA prepared by the detergent method. They have a coiled, highly contracted configuration. This is indicative of a large amount of intramolecular bonding since without this a flexible polyelectrolyte such as RNA would be highly extended, exhibiting a high viscosity and low sedimentation constant rather than the reverse.

(iii) The dissociation of the RNA components into subunits which is brought about by heating appears to involve the breaking of internucleotide hydrogen bonds. These bonds are considered to be grouped into short sequences that may unite regions of the same chain or of two different chains.

(iv) The degree of hydrogen-bonding in the heated RNA is indistinguishable from that in the unheated material, both with respect to the number of hydrogen bonds present and to their thermal stability.

The general outline of the macromolecular organization of RNA which these findings provide is as follows: two components of RNA isolated by the phenol method are composed of approximately 10 and 5 polynucleotide chains, joined in each case to one another by a small number of internucleotide hydrogen bonds. In the single chain regions between these junctions, the chains are coiled in such a way as to permit further hydrogen bonding between nucleotides in the same chain.

The temperature sensitivity of the ultraviolet absorption of RNA at 25°C (Fig. 2) is evidence that the weakest hydrogen-bonded regions are unstable even at this temperature. This instability presumably accounts for the slow release of subunits as seen in the gradual fall of the viscosity at 25°C. This structural instability in the RNA at room temperature is surprising since the microsomal particles are quite stable. (If the particles are functioning as templates in protein synthesis a stable, ordered structure is an absolute necessity.) These considerations taken together with the obvious concern as to how the RNA is arranged in the microsomal particle led us to examine the state of hydrogen-bonding of the RNA in these particles.

4. Studies of Microsomal Particles

(a) *Viscosity and sedimentation properties of microsomal particles*

The ultracentrifuge pattern of calf liver microsomal particles (Fig. 6) consisted of four peaks. These appear to correspond to the components A, B, C and E observed by Petermann & Hamilton (1956) in rat liver microsomal particles, both as regards the rates of sedimentation and the relative areas under the peaks. The sedimentation coefficient of the major component was measured as a function of concentration. The extrapolated value of the sedimentation coefficient at zero concentration is 81·2 *S*. The slope of the plot was between zero and 0·03, indicative of a slight concentration dependence.

The reduced specific viscosity of microsomal particles was measured at concentrations of 0·8 and 0·9 g/100 ml. in 0·02 M-phosphate buffer. The value of η_{sp}/c was 0·039 in both cases. In general, the amount of dependence of η_{sp}/c upon concentration varies as the square of the intrinsic viscosity (Huggins, 1942). Because the viscosity of the microsomal particles was quite small and was the same at two concentrations, the measured reduced specific viscosity and the intrinsic viscosity are essentially the same. In units of dl./ml., the intrinsic viscosity is 0·060 as compared to the theoretical value of 0·025 for unhydrated spheres. Provided the shape is spherical, the apparent hydration is about 1 g water per g of nucleoprotein.

The intrinsic viscosity and sedimentation coefficient may be used to estimate the

molecular weight of the microsomal particles, provided the shape is known. The large sedimentation coefficient and small intrinsic viscosity of microsomal particles are indicative of a very small axial ratio. There are as yet no experimental grounds for assuming the shape of the particles to be other than spherical. For spheres the general

Fig. 6. The sedimentation pattern of liver microsomal particles. Photograph taken after 25 minutes at 27,020 rev/min with bar angle at 22°.
Solvent: 0·02 M-phosphate buffer.
Concentration: 0·9 g/100 ml.

equation of Scheraga & Mandelkern (1953), referred to in Section 3a, is valid with $\beta = 2{\cdot}1 \times 10^6$. In this case, the equation is simply the Einstein-Stokes relation. Assuming additivity of the volumes occupied by proteins and RNA in the microsomal particles and an RNA content of 40 %, $\bar{v}$ is calculated to be 0·67. A molecular weight of 3,700,000 for the microsomal particle is then obtained from the Einstein-Stokes equation.

(b) *Temperature-dependence of the ultraviolet absorption and formaldehyde reactivity of microsomal particles*

We have made a preliminary study of the effect of increasing temperature upon the ultraviolet absorption of microsomal particles and upon their reactivity with formaldehyde, in order to estimate the extent and stability of internucleotide hydrogen-bonding in the particles.

Our interpretation of these results is very tentative because they are not supported by the optical rotation studies made on RNA, and the existence of protein offers a new source of interaction with RNA that may affect the ultraviolet absorbance and formaldehyde reactivity in a way similar to base pairing.

Upon measuring the optical density as a function of temperature it was found that no changes occurred in the 0·02 M buffer until about 55°C. Above this temperature the optical density rose but the complete profile could not be obtained in the available temperature range. This became possible, however, when the buffer concentration

was reduced to 0·001 M-phosphate: the results are shown in Fig. 7. In this same solvent RNA itself would have an optical density-temperature profile lying at considerably lower temperatures. The comparison at the same ionic strength is not warranted, however, because of the much greater net charge of the RNA and the lowering of the melting out which this causes. At the higher ionic strength of 0·02 M-phosphate the optical density-temperature profile is moved to higher temperatures so that its upper part

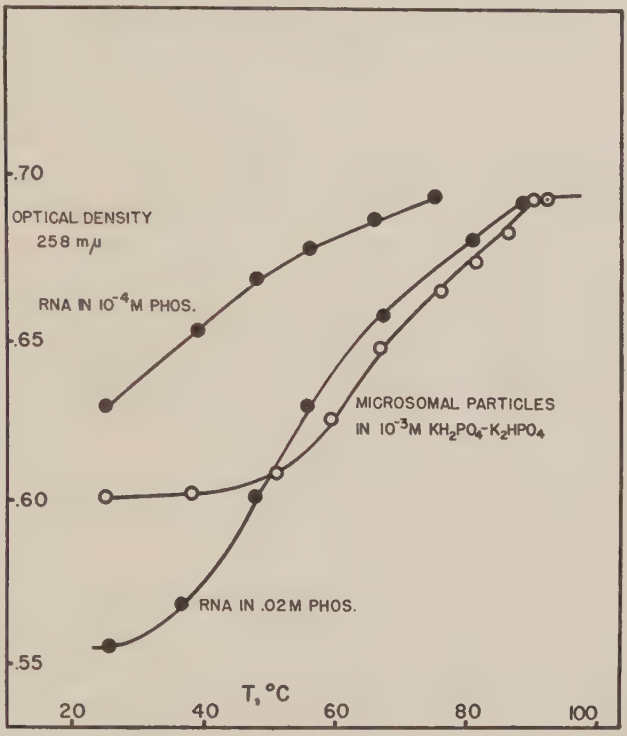

FIG. 7. The temperature-dependence of the optical density of microsomal particles and RNA.

nearly coincides with that of the microsomal particles. This result is included in Fig. 7. Comparison shows that the first third of the transition that occurs in RNA is missing in the microsomal particles, but the remainder coincides. This preliminary experiment may be tentatively interpreted as indicating that the helical content of RNA in the microsomal particles is only two-thirds as much as in isolated RNA, but that the additional helical regions in isolated RNA are particularly weak. They may indeed arise in a non-specific way after removal of the protein.

Equally preliminary studies of formaldehyde reactivity do support the above interpretation. In these, it was observed that in 0·001 M buffer a temperature of 80°C was required to bring about complete reaction in the particles in 10 hr, whereas only a temperature of 55°C was required for RNA. When the reactivity was measured as a function of temperature, a marked increase was found to set in at about 55°C for the particles. Thus we have these two indications of base pairing and helical arrangement in microsomal particles. Specifically, these show that about 25 % of the nucleotides are in helical regions and that these are not subject to thermal disruption below 50°C in 0·001 M salt and higher temperatures at higher ionic strengths. If these tentative conclusions prove true, it would appear that the helical regions are an important

aspect of the molecular architecture of microsomal particles and that their functional role deserves investigation.

As further investigation of microsomal particle structure continues, it may prove useful to keep in mind two possibilities suggested by the present work. One is that the dissociation of the 80 *S* microsomal particles into two smaller particles (62 *S* and 47 *S*) may correspond to a cleavage that separates the regions of the parent particle that contain the two RNA components isolated by the phenol method. Unfortunately, we have been unable to demonstrate this in preliminary experiments, but the possibility of doing so remains. Likewise, the further disintegration of the particles into still small but uniquely definite units may correspond to the breaking of the next weakest secondary linkages within the RNA components. This too is open to direct exploration. The second observation is that the absence of the weaker fraction of inter-nucleotide hydrogen bonds in the particles, relative to the isolated RNA, suggests that these sites may be available in the particle matrix for bonding with soluble RNA or to function in some other way in the assembly of protein molecules.

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J. Mol. Biol. (1959) **1**, 127-133

Etude par diffusion des rayons X aux petits angles des gels d'acide désoxyribonucléique et de nucléoprotéines : (note préliminaire)

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(*Received 16 January 1959*)

Deoxyribonucleic acid (DNA), nucleoprotamine (DNP) and nucleohistone (DNH) have previously been investigated by means of X-ray diffraction techniques, the samples being oriented fibres in equilibrium with an atmosphere of known humidity.

We have now studied the low-angle X-ray scattering from unoriented aqueous gels of these three substances, as a function of the concentration of dry material and of electrolyte. We have investigated the phase diagrams of these systems, and have made observations on unfixed nuclei containing DNP and DNH. This technique permits the investigation of long-range organization, and the use of concentrations and ionic strengths in the physiological range. DNA and DNH were extracted from fowl erythrocytes, DNP from trout sperm.

The DNA-water system contains one phase in the range between 5 % and 50 % DNA (weight of DNA per weight of gel); the structure of this phase seems to consist of rigid rods, packed in a hexagonal array and swollen by water. The mass per unit length of the rods is in good agreement with Crick & Watson's model of DNA.

The behaviour of the DNP water system is quite different. At all concentrations from 5 % to 50 % the system contains two phases, namely the saturated solution and a paracrystalline phase. At higher concentrations only the paracrystalline phase exists; the diffraction lines of this phase are very sharp. The X-ray photographs of unfixed trout sperm heads and of the paracrystalline phase of DNP are identical.

In the DNH-water system there exist several phases. At very low concentrations there is only a solution of DNH in water which gives a diffuse central spot in the X-ray diagram. At higher concentrations (about 30 %) there is a phase which we denote ω: its characteristic diffraction diagram is formed of three bands, at spacings 110 Å, 55 Å, and 37 Å. At a concentration of 50 % is found another phase (denoted ψ): its X-ray diagram contains one band at a spacing $\sim$ 30 Å. At even higher concentrations ($\sim$ 80 %) a fourth phase appears (phase χ); it gives two fairly sharp bands at spacings of 80 Å and 40 Å. Each phase exists over a small concentration range: the X-ray diagrams vary slightly with the concentration. At all concentrations intermediate between two phases the X-ray diagrams indicate the coexistence of the diagrams of both phases. The X-ray photographs of unfixed fowl erythrocyte nuclei show the three typical bands of phase ω of DNH; the structure of DNH in the nuclei therefore seems to be identical with phase ω.

The behaviour of DNP-water and DNH-water systems suggests that several DNA molecules are linked together by the basic proteins and form a three-dimensional complex which can be swollen by water only to a limited extent. In contrast, DNA can be swollen by water to any extent in the absence of basic proteins.

The structure of the DNP paracrystalline phase is very compact and highly organized, and contains only 35 % water. On the other hand, the ω phase of DNH is poorly organized; it contains over 60 % water and it shows long-range order. This difference between DNP and DNH can be interpreted in terms of the biological role of these substances.

The X-ray diagram of the ω phase of DNH is compatible with a lamellar structure having a period of 110 Å. Some electron microscope observations seem to confirm the lamellar structure, but this model needs further confirmation.

The action of deoxyribonuclease on DNH gels shows that the observed features must be attributed to a DNA-containing phase, and not to a lipid impurity as has been previously suggested.

1. Introduction

L'étude cristallographique des fibres d'acide désoxyribonucléique (ADN) est poursuivie depuis plusieurs années dans de nombreux laboratoires: Crick & Watson (1954) ont proposé un modèle de structure, devenu désormais classique, qu'ont pleinement confirmé les cristallographes (Franklin & Gosling, 1955; Langridge *et al.*, 1957).

Les fibres de nucléoprotamines (DNP) et nucléohistones (DNH) ont été surtout étudiées par l'équipe de King's College. Ces chercheurs (Wilkins, 1956) ont montré que les fibres de DNP ont une structure assez bien organisée: les diagrammes de diffraction sont riches en raies fines, orientées par rapport à l'axe de la fibre. Ces diagrammes leur ont suggéré un modèle de structure, formé d'hélices d'ADN, autour desquelles s'enroule la protamine, qui comble le moins profond des deux sillons qui parcourent la surface de l'hélice. Les diagrammes de diffraction des fibres de DNH sont bien moins nets que ceux des DNP: on reconnaît à peine quelques bandes diffuses, peu orientées par rapport à l'axe de le fibre. Wilkins et ses collaborateurs (1956) ont interprété ces diagrammes en proposant un modèle de structure analogue à celui des DNP, auquel ils ont ajouté une deuxième chaîne polypeptidique enroulée autour de l'hélice d'ADN, logée cette fois dans le sillon le plus profond.

En fait, si ces modèles sont compatibles avec les diagrammes de diffraction, les données expérimentales sur lesquelles ils sont fondés ne sont pas assez riches et nettes pour que les structures proposées soient tout-à-fait convaincantes, comme l'est, au contraire, la structure de l'ADN décrite par Crick & Watson.

Nous nous sommes alors proposés d'apporter au problème de la structure de DNP et DNH d'autres données expérimentales: nous avons entrepris l'étude systématique de la diffusion des rayons X aux petits angles par des gels aqueux, en fonction de la concentration en ADN, DNP ou DNH, en variant la teneur en électrolyte (ClNa). (Riley & Oster (1951) ont effectué un travail analogue avec l'ADN.)

Cette technique permet d'examiner l'organisation qui existe à une échelle intermédiaire entre les dimensions moléculaires ($\sim$ 20 Å) et $\sim$ 200 Å. En outre on étudie ainsi des échantillons à des concentrations en eau et électrolyte voisines des conditions physiologiques: en examinant la diffusion des rayons X aux petits angles produites par des noyaux entiers et intacts, nous avons pu reconnaître certaines structures qui existent dans les gels préparés avec les nucléoprotéines extraites.

Nous avons décrit dans une courte note quelques résultats préliminaires (Luzzati & Nicolaïeff, 1959).

2. Partie Expérimentale

L'étude détaillée a été effectuée avec ADN et DNH extraites de noyaux d'érythrocytes de poulet, et avec DNP extraite de sperme de truite. Nous avons examiné également

quelques échantillons extraits d'organes d'autres animaux: nous décrirons ultérieurement les résultats de ces expériences. Les extractions ont été effectuées par M. Vendrely et Mme Mazen-Knobloch par la méthode saline (Vendrely, Knobloch, Vendrely & Matsudaïra, 1959): M. Vendrely a également effectué pour nous des analyses chimiques. Les noyaux d'érythrocytes de poulet et les têtes de spermatozoïdes de truite ont été isolés par Mme C. Vendrely (Vendrely *et al.*, 1959).

Nous avons préparé les échantillons en mélangeant des fibres, séchées à l'air (dont la teneur en H_2O est connue) avec de l'eau, pure ou salée, et en les laissant reposer jusqu'à ce que l'équilibre soit atteint (cela peut exiger plusieurs semaines). Une trace de thymol évite les contaminations bactériennes.

Les gels sont ensuite enfermés dans des cuves rectangulaires en plexiglas d'un millimètre d'épaisseur, dont 2 faces sont constituées par des feuilles minces de mica, transparentes aux rayons X: l'étanchéité des cuves est assurée par un serrage mécanique. Les échantillons ainsi préparés et enfermés dans les cuves ne sont pas orientés; ils sont cependant fortement biréfringents. La concentration est vérifiée après chaque expérience par un dosage de phosphore. Nous avons constaté que les fibres contiennent au départ une quantité, petite mais variable, de ClNa: la teneur en sel n'a donc pas pu être déterminée avec précision.

Nous avons utilisé une chambre de diffraction, construite au laboratoire, sur le principe des chambres à focalisation type Guinier. Le rayonnement rigoureusement monochromatique ($K\alpha_1$ du cuivre) est focalisé à 50 cm environ du monochromateur: un système de fentes linéaires définit un faisceau haut et étroit, et limite la région centrale souillée. Nous avons exploré la région du diagramme de diffraction comprise entre $s = 1/200$ Å^{-1} et $s = 1/10$ Å^{-1} environ ($s = 2 \sin\theta/\lambda$). La cuve porte-échantillon, ainsi que les fentes et le film photographique sont enfermés dans une enceinte dans laquelle on fait le vide. Les temps de pose varient entre 5 et 48 heures.

Les concentrations sont indiquées en poids de ADN, DNP ou DNH par rapport au poids total du gel.

3. Acide Désoxyribonucléique

Nous avons examiné l'ADN en solution dans l'eau pure et salée (jusqu'à ClNa 1 molaire), à des concentrations allant de 5 % à 50 % en poids d'ADN: à des concentration plus élevées la viscosité empêche la manipulation des échantillons, et surtout il devient difficile d'éviter l'orientation spontanée. D'ailleurs vers $c = 50$ % on atteint la concentration des fibres étudiées par Franklin, Wilkins et leurs collaborateurs (Feughelman *et al.*, 1955).

Aux faibles concentrations on observe seulement une tache centrale diffuse. Lorsque la concentration augmente, l'intensité de cette tache diminue, et il apparaît une bande aux environs de $s = 1/40$ Å^{-1}, floue d'abord, qui s'affine aux concentrations les plus elevées. La position de la raie varie avec la concentration; la loi de variation de l'espacement de Bragg est celle d'un arrangement hexagonal de bâtonnets cylindriques très longs, séparés par le solvant (Luzzati, Mustacchi & Skoulios, 1958).

$$c = \frac{m/l}{\frac{m}{l} + \left(\frac{2}{\sqrt{3}} d^2 - \frac{m}{l\delta_{\mathrm{ADN}}}\right)\delta_{\mathrm{H_2O}}},$$

où m/l est la masse des bâtonnets par unité de longueur, δ_{ADN} et $\delta_{\mathrm{H_2O}}$ sont la densité de l'ADN et du solvant, d est l'espacement de Bragg de la raie $(10\bar{1}0)$ du réseau hexagonal, celle qu'on observe expérimentalement. En mesurant c et d, on peut calculer m/l: on obtient, à toute concentration, $m/l \approx 3{\cdot}3 \times 10^{-22}$ g/Å en bon accord avec le modèle de Crick & Watson.

La teneur en sel ne joue pas un rôle important dans ce système, bien que la forme des raies aux faibles concentrations en ADN et le diamètre des bâtonnets varient

légèrement avec la concentration du sel. C'est un point sur lequel nous nous proposons de revenir ultérieurement.

4. Nucléoprotamine

Nous avons examiné des gels des DNP dans l'eau et dans l'eau salée (ClNa 0·14 et 1 molaire), à des concentrations allant de 5 % à 50 % environ.

Aux faibles concentrations on observe une tache centrale diffuse. A mesure que la concentration augmente, l'intensité de cette tache diminue, et il apparaît d'abord un ensemble de raies fines, dont l'intensité augmente ensuite. La position et la forme de ces raies ne varient pas jusqu'aux concentrations les plus fortes (50 % environ) (Plate I). Nous n'avons pas poursuivi l'exploration au delà de cette concentration, où on atteint le domaine étudié par Wilkins et ses collaborateurs (Feughelman *et al.*, 1955). La position de la première raie est $s = 1/27$ Å^{-1}.

Nous avons comparé ce diagramme de diffraction avec celui d'une préparation de têtes de spermatozoïdes de truite: ils sont identiques, ce qui confirme les résultats d'autres chercheurs (Wilkins, 1956).

La teneur en sel ne semble jouer aucun rôle dans ce système, sauf aux concentrations les plus faibles.

5. Nucléohistone

Nous avons examiné des gels de DNH dans l'eau pure et salée (ClNa 0·14 et 1 M), à des concentrations allant de 5 % à 80 % environ. La valeur absolue des concentrations supérieures à 50 % n'a pas pu être déterminée avec précision.

Aux concentrations faibles, une tache de diffusion centrale entoure le faisceau direct. A des concentrations plus grandes l'intensité de cette tache diminue et il apparaît un système de 3 bandes assez floues, mais bien définies, dont la position correspond aux trois premiers ordres d'un espacement $d \sim 120$ Å. Ces espacements diminuent ensuite légèrement lorsque la concentration augmente tandis que les rapports entre les intensités des trois bandes changent nettement: la position moyenne des trois bandes est $s_1 = 1/110$ Å^{-1}, $s_2 = 1/55$ Å^{-1}, $s_3 = 1/37$ Å^{-1}. Nous appelons ω la structure caractérisée par ce système de bandes (Plate II *a*).

A des concentrations supérieures à ~ 35 %, en plus de ces trois bandes il apparaît un autre type de diagramme, qui devient prédominant vers 50 %, concentration à laquelle les bandes de la structure ω ont disparu. Ce diagramme est caractéristique d'une structure que nous appellerons ψ; il est formé d'une bande assez nette, située à $s \sim 1/30$ Å^{-1}, dont la position varie légèrement avec la concentration (Plate II *b*).

A des concentrations encore plus élevées il apparaît une troisième structure (structure χ), caractérisée par deux bandes dont les positions sont celles des deux premiers ordres d'un espacement de 80 Å ($s_1 = 1/80$ Å^{-1}, $s_2 = 1/40$ Å^{-1}) (Plate II *c*). Nous n'avons pas pu déterminer avec précision le domaine de concentration auquel correspond la structure χ: nous estimons qu'il se trouve aux environs de 80 %.

Le diagramme obtenu avec les noyaux d'érythrocytes entiers est identique à celui de la structure ω: la structure responsable de ce système de trois bandes semble donc être celle qui existe dans le DNH à l'intérieur du noyau (voir à ce sujet Wilkins, 1956).

Dans ce système, comme dans la DNP, le rôle de la teneur en sel est peu important: tout au plus la composition exacte de chaque phase semble en dépendre, mais non leur structure.

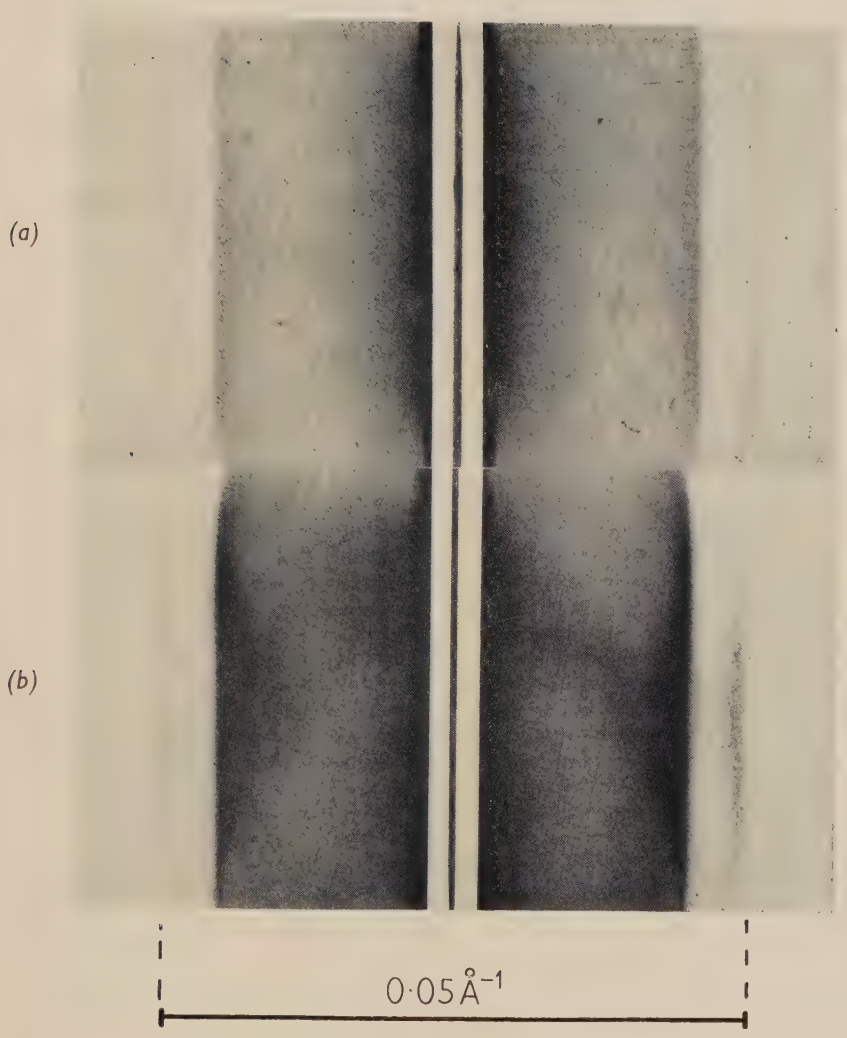

PLATE I. Gels de nucléoprotamine de sperme de truite.
(a) $c = 9$ % dans l'eau.
(b) $c = 48$ % dans l'eau.

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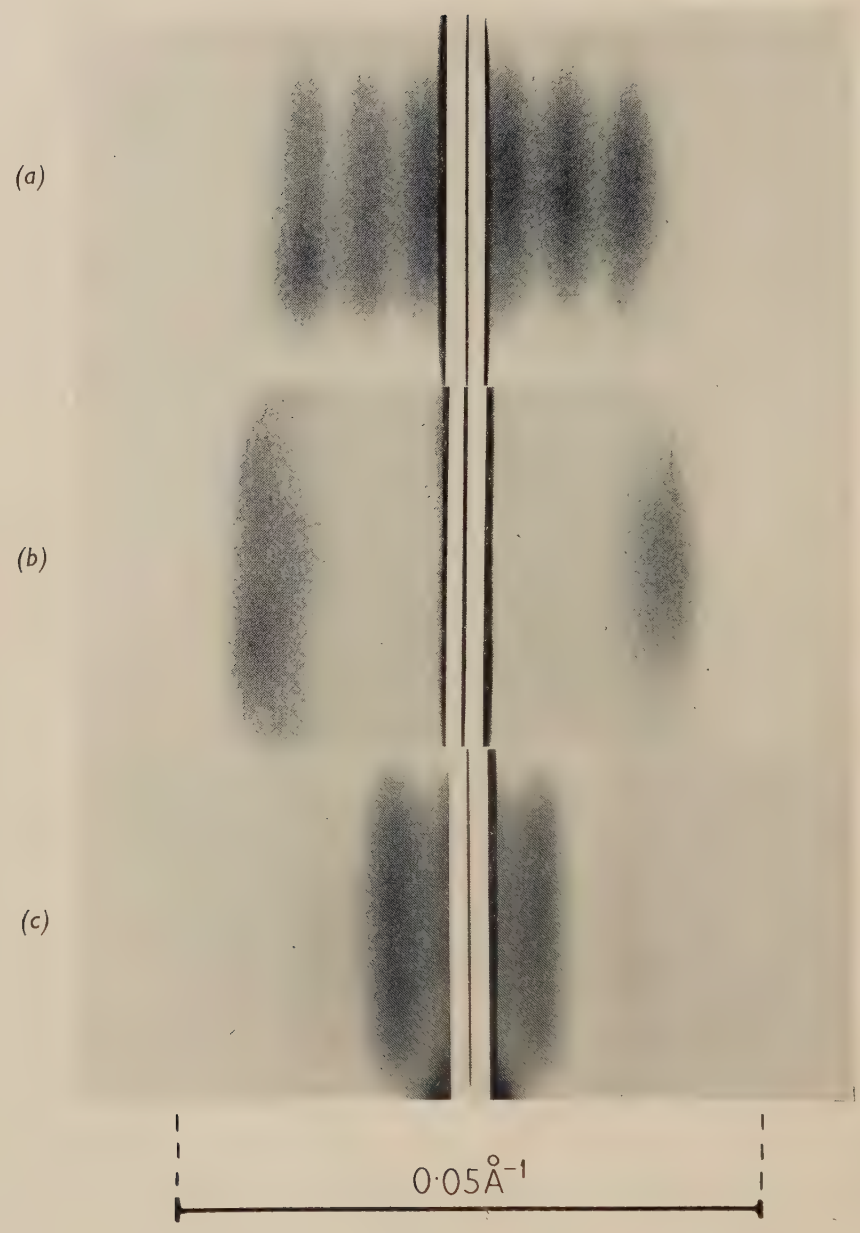

PLATE II. Gels de nucléohistone d'érythrocyte de poulet.
(a) $c = 27$ % dans l'eau salée (Cl_2Mg, 0·1 M).
(b) $c = 52$ % dans l'eau salée (ClNa, 0·15 M).
(c) $c = 83$ % dans l'eau salée (ClNa, 0·15 M).

6. Discussion

Les résultats expérimentaux décrits ci-dessus mettent en évidence une différence structurale importante entre les gels d'ADN, de DNP et de DNH. Le système ADN-eau contient à chaque concentration une seule phase: dans un domaine étendu en concentration toute l'eau est donc logée entre les molécules d'ADN. Au contraire la phase paracristalline des DNP contient une quantité fixe d'eau: l'eau en excès entre dans une phase plus hydratée (vraisemblablement la solution saturée, responsable de la diffusion centrale) qui existe en équilibre avec la phase paracristalline.

Dans le système DNH-eau les deux structures ω et ψ semblent correspondre à deux phases distinctes, car apparemment le passage entre les deux structures se fait par une zône de démixtion, où les deux phases coexistent en équilibre. En plus il existe probablement une phase plus hydratée (la solution saturée), et une phase très concentrée, responsable du diagramme du type χ. Le domaine d'existence de chacune des phases s'étend sur un petit intervalle de concentration.

En fait cette différence entre ADN, DNP et DNH est nette seulement aux concentrations modérément élevées; aux fortes concentrations ($c > 50$ %) d'une part dans l'ADN il apparaît une autre phase (forme A cristalline), et d'autre part les dimensions du réseau de la DNP varient avec la concentration (Feughelman, 1955), ce qui indique que le système ne contient plus qu'une seule phase.

Si nos résultats n'apportent pas de renseignements nouveaux quand à la structure de l'ADN, il n'en est pas de même en ce qui concerne DNP et DNH.

Le comportement des systèmes binaires DNP-eau et DNH-eau semble indiquer que les molécules protéiques relient entr'elles plusieurs molécules d'ADN, et cela vraisemblablement par les liaisons entre les phosphates et les acides aminés basiques; il se formerait ainsi des réseaux à trois dimensions, dont le taux d'hydratation ne peut varier qu'entre d'étroites limites.

Au contraire il semble peu probable que les gels contiennent de véritables molécules de nucléoprotéine, dans lesquelles chaque chaîne polypeptidique est liée à une seule molécule d'ADN, car dans ce cas les gels d'ADN et ceux des nucléoprotéines devraient avoir le même comportement, du moins aux faibles concentrations où les molécules ne seraient pas en contact: c'est une objection qu'on peut faire aux modèles proposés par Wilkins *et al.* (1956).

Il est en outre manifeste que la structure des DNH, aussi bien dans les noyaux intacts que dans les gels, est à la fois bien moins ordonnée (ce que montre la forme des raies de diffraction), et très différente de celle des DNP. Il est remarquable que seulement dans les DNH il existe une organisation à l'échelle des 100 Å, donc nettement plus grande que les dimensions transversales de la molécule d'ADN. Cela est à mettre en rapport avec le rôle biologique de ces substances. En effet la DNP existe seulement dans le sperme de certaines espèces où l'ADN ne semble pas jouer un rôle métabolique important: il s'agit plutôt d'un réservoir qui assure le transport du matériel génétique jusqu'à l'ovule. Cette fonction est compatible avec la structure compacte et organisée des DNP. Au contraire les DNH des noyaux somatiques ont une fonction métabolique qui s'effectue vraisemblablement par une circulation de matière entre la DNH et le milieu environnant: une structure ouverte et peu organisée semble être mieux adaptée à cette fonction. Remarquons, à ce sujet, que la DNH, forme ω, contient au moins 65 % d'eau: la forme paracristalline de la DNP n'en contient que 35 % environ.

Des trois formes de DNH que nous avons reconnues, la forme ω est particulèrement

importante, car elle existe dans les noyaux intacts. Elle est caractérisée par trois raies de diffraction, correspondant aux espacements 110 Å, 55 Å et 37 Å. Or cette suite de raies équidistantes suggère une structure lamellaire (Luzzati *et al.*, 1958), de périodicité égale à 110 Å. Il peut paraître surprenant que les molécules cylindriques comme celles d'ADN s'organisent en feuillets lorsqu'elles s'associent avec les histones; en fait il ne serait pas difficile de proposer des modèles lamellaires compatibles avec la structure connue de l'ADN. Nous préférons remettre à plus tard le choix du modèle, en attendant d'autres données expérimentales.

Une structure lamellaire de période égale ou voisine à 110 Å a été observée au microscope électronique dans les noyaux de spermatocytes de certains mollusques et insectes (Grassé, Carasso & Favard, 1956; Rebhurn, 1957; Gall & Bjork, 1958): bien qu'il s'agisse de noyaux d'un type spécial, la coïncidence avec nos résultats est frappante.

Pour terminer nous tenons à lever un doute exprimé par Wilkins (1956): selon lui une raie correspondant à $s = 1/60$ Å^{-1}, qu'il a trouvée dans certaines préparations, pourraît être dûe à une impureté lipidique. M. Vendrely, qui a effectué les analyses chimiques relatives à la DNH utilisée dans cette étude, exclut que la teneur d'une éventuelle impureté lipidique puisse dépasser 5 %. Or, même en admettant que l'échantillon contient 5 % de lipides, et même si ces lipides étaient parfaitement organisés, les raies de diffraction observées sont beaucoup trop intenses pour pouvoir être dues à ce résidu lipidique: c'est ce que montrent quelques mesures absolues de diffusion des rayons X. En outre, nous avons fait agit la désoxyribonucléase sur les gels de DNH ayant la structure ω: le système de trois bandes disparaît entièrement. Il est donc exclu que dans notre cas une phase lipidique séparée puisse être responsable des diagrammes de diffraction. Il est possible toutefois que des faibles quantités de lipides participent à la structure de la forme ω: si c'était le cas, l'intérêt des observations rapportées ci-dessus ne serait nullement compromis puisqu'il ne s'agirait pas d'un artéfact, la même structure existant dans les noyaux.

Note ajoutée aux épreuves.

La DNH utilisée a été extraite par l'action de l'eau salée (ClNa, 1 M). Par ailleurs nous avons examiné récemment des échantillons de DNH d'érythrocytes de poulet extraite à l'eau, et plusieurs autres échantillons de DNP et DNH provenant d'organes différents de plusieurs espèces animales (Nicolaïeff, Mazon-Knobloch, Vendrely & Luzzati, 1959): l'ensemble des observations confirme les résultats décrits dans cette note.

Nous tenons à remercier Mme et M. Vendrely et Mme Mazen-Knobloch de leur précieuse collaboration et le Dr. M. H. F. Wilkins pour ses critiques et la communication de résultats non publiés.

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Dispersal of the Parental Nucleic Acid of Bacteriophage T4 Among its Progeny†

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The distribution of the parental deoxyribonucleic acid (DNA) passed on from T4 bacteriophages to their offspring has been measured by allowing radioactive decay to proceed in first generation progeny phages containing radiophosphorus atoms transferred to them from highly ^{32}P-labeled parental viruses. The amount of once-transferred parental radioisotope transferable from the first generation progeny to their own second-generation progeny was then measured at various stages of radioactive decay, in order to determine the lethal effects produced by ^{32}P disintegrations within those first generation progeny particles which carry the parental atoms. It was found that whereas before decay about half of the parental ^{32}P harbored by the first generation progeny can be once more transferred to the second generation progeny, subsequent radioactive disintegrations in the first generation progeny rapidly reduce the transferable radio-isotope to one-third of the initial value. This final third of the parental ^{32}P remains transferable, however, even after further extensive radioactive decay has been allowed to take place. Quantitative evaluation of the observed rate of loss of transferability with decay indicates that approximately 60% of the transferred parental DNA appears in the first generation progeny in aggregates each amounting to 15% of the total DNA complement per phage and that the remainder is dispersed into small fragments no greater than 0·3% of the amount of DNA per phage.

This dispersal of the parental nucleic acid does not seem to be produced by random breakage forces acting on the DNA in the course of intracellular phage reproduction. For it was found that the distribution of the parental isotope among the first generation progeny remained unaffected if the bacteria infected with the ^{32}P-labeled parent phages were temporarily exposed to chloramphenicol. Under these conditions, phage DNA synthesis proceeds while formation of intact progeny phages is held in abeyance until removal of the antibiotic. Hence if the viral DNA were subject to the action of random breakage forces during its replication, this artificial extension of the eclipse should have caused a greater dispersal of the transferred atoms. The distribution of parental isotope, furthermore, was also studied among the *second* generation progeny by carrying out third generation transfer experiments with aliquots of second generation progeny at various stages of radioactive decay. It was found that the transferable fraction of the twice-transferred parental isotope decreased with radioactive decay in the same manner as that of the one-transferred label in the first generation progeny. This indicates that after a second transfer cycle the distribution of the parental atoms remains essentially unchanged, in contrast to the anticipated further dispersal if random breakage forces obtained.

The observed distribution of the transferred DNA atoms must, therefore, reflect an *a priori* division of the nucleic acid of T-even phages into parts which are destined to be transferred in large and small fragments respectively. These results are in agreement with those obtained by Levinthal (1956) through direct autoradiography.

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1. Introduction

At the outset of the intrabacterial multiplication cycle, infecting T-even† bacteriophage particles introduce their deoxyribonucleic acid (DNA) into the host cell (Hershey & Chase, 1952). Approximately half of the atoms of the parental DNA subsequently reappear again among, or are "transferred" to, the several hundred progeny virus particles liberated upon lysis of each infected bacterium (Putnam & Kozloff, 1950; Watson & Maaløe, 1953). The fact that no more than half of the parental DNA atoms are transferred can be attributed to the additive effect of a number of technical difficulties inherent in the transfer measurements themselves, rather than to some more profound aspect of intracellular phage multiplication (Hershey & Burgi, 1956). One may suppose, therefore, that those atoms which are passed on from parent to progeny constitute a representative sample of the DNA molecules which initiated a successful infection. In order to gain some insight into the problem of how the hereditary structures residing in the parental phage DNA may go about their manifold reduplication in the course of bacteriophage growth, it appears desirable to know how atoms transferred from parental to progeny DNA are *distributed* over the progeny particles, i.e. to what extent the integrity of the parental DNA has been conserved or destroyed. This knowledge is useful since an important operational distinction between different conceivable DNA replication mechanisms is the distribution of the atoms of the parental molecule over its replicas (Levinthal, 1956; Delbruck & Stent, 1956; Meselson & Stahl, 1958). It has been shown previously that the parental T-even bacteriophage DNA is, in fact, dispersed over several of its progeny particles, whose number was estimated to be between 8 and 25 (Stent & Jerne, 1955). In these experiments the distribution of the transferred DNA atoms was estimated by means of the lethal effects on the progeny population of the decay of incorporated radiophosphorus ^{32}P atoms derived from the DNA of highly ^{32}P-labeled parental bacteriophages. It is the purpose of this report to present an extension of this work, in which the nature of the dispersal of the DNA patrimony among the progeny viruses has been studied in more detail.

2. Materials and Methods

Bacteriophage. The tryptophan-requiring strain T4.38 (Wollman & Stent, 1950).

Bacteria. Escherichia coli, strains B/r (Witkin, 1951), BR2 (Hershey, Dixon & Chase 1953), BB (the "Berkeley" strain derived from strain B), and B/4 (a T4-resistant mutant of strain B).

Media. H medium is the glycerol-lactate medium previously described (Stent & Fuerst, 1955), except that the present H medium contains no peptone and its 0·5 g/l. casein hydrolysate consists of a 1:1 mixture made from ordinary casamino acids DIFCO (which supply the total phosphorus content of the medium of 2·5 mg/l.) and a phosphorus-free casamino acid preparation from which the phosphorus has been removed by precipitation as the magnesium ammonium phosphate. *Tryptone broth* (TB) contains 8 g bacto-tryptone DIFCO and 5 g NaCl per liter of distilled water. *Salt-poor TB* has the same composition as TB, except that no NaCl is added.

Infectivity assays. The number of viable T4 phages was assayed by the soft agar layer technique on nutrient broth agar plates seeded with strain BB.

Radioactive techniques. The measurement of specific radioactivity was carried out as previously described (Stent & Fuerst, 1955). Carrier-free radiophosphorus was supplied by the Radiochemical Centre, Amersham, England.

† The two related coliphage strains T2 and T4 will be referred to collectively as "T-even."

3. Theory

Bacteriophages containing a high specific activity of incorporated radiophosphorus ^{32}P lose their infectivity by decay of the radioactive atoms. The kinetics of inactivation proceed in such a manner that the fraction of surviving phage particles, s, decreases with the fraction f of ^{32}P atoms which have decayed by the time of assay according to

$$s = e^{-N^*\alpha f} \tag{1}$$

where N^* is the initial number of ^{32}P atoms per phage particle and α the fraction of radioactive disintegration which are lethal to the phage in which they occur. For the T-even bacteriophages, α has the value of 0·1 at 4°C. It is, therefore, possible to estimate the ^{32}P content of a *homogeneous* population of uniformly labeled phages by measurement of their inactivation rate (Hershey, Kamen, Kennedy & Gest, 1951). In case of *heterogeneous* phage population of which a fraction g_i of the particles is initially labeled with N_i^* atoms of ^{32}P per phage, overall inactivation will proceed according to

$$s = \sum_i g_i e^{-N_i^*\alpha f} \tag{2}$$

A plot of log s against f according to (2) results in a concave survival curve, from whose initial and final slopes the rate of inactivation, and hence the specific radioactive content, of the most and least highly ^{32}P labeled members of the population respectively can be estimated. Hence the distribution of ^{32}P isotope of a heterogeneously labeled phage population can be determined by resolving its complex survival curve in terms of equation (2). But if the population contains a major fraction of phages which possess no ^{32}P at all, there will be no appreciable death, and the ^{32}P content of the radioactive minority cannot be measured in this way. One may, however, measure the effect of radioactive decay on the viability of a homogeneously ^{32}P-labeled phage population not only by counting the number of infective virus particles but also by determining the amount of radioisotope which this population can still transfer in *single* infection to its progeny. For about half of the parental ^{32}P atoms are transferred to the progeny DNA as long as all the virus particles are infective; but when only a fraction s of the phages survive radioactive decay, the amount of transfer is reduced by the same factor s (Stent & Jerne, 1955). In case of a heterogeneous phage population in which a fraction p_i of the ^{32}P isotope resides in particles initially labeled with N_i^* atoms of ^{32}P per phage, the transferable fraction T of the radioactivity in the population thus decreases with radioactive decay from its initial value of T_0 according to

$$T = T_0 \sum_i p_i e^{-N_i^*\alpha f} \tag{3}$$

It is, therefore, possible to estimate the distribution of isotope in a heterogeneously labeled population also by carrying out transfer experiments with the phage at various stages of radioactive decay and resolving the resulting transfer survival curve in terms of equation (3). The advantage of this much more laborious method is that the presence of a large fraction of phages containing no radioisotope at all does not interfere, since such particles, though not subject to any inactivation by decay, also do not contribute anything to the transfer measurements (i.e., $p_j = 0$ for $N^* = 0$).

4. Experimental

Previous studies have already indicated that most of the phosphorus transferred from the parental T-even DNA must be concentrated in a few offspring individuals

(Stent & Jerne, 1955). Hence the progeny of uniformly labeled parents represent precisely such a heterogeneously labeled phage population in which a minority of the particles are much more highly radioactive than the majority. Since the first generation progeny possessing ^{32}P transferred to them from uniformly labeled parents again pass on about half of their isotope to their own, second generation, progeny (Maaløe & Watson, 1951), it would be possible to determine the distribution of parental radioactivity among the first generation progeny by means of the transfer-decay method, provided that the decay of transferred ^{32}P inactivates phage particles with an efficiency similar to that of the decay of ^{32}P atoms built into the viral DNA directly from the phosphorylated constituents of host cell or growth medium. The plan of the present experiments, therefore, was to grow highly ^{32}P-labeled parental stocks of T4, and immediately to infect bacteria with these phages to produce first generation progeny populations containing the transferred radioisotope. These progeny were then stored and second generation transfer experiments carried out from time to time with aliquots of this population in order to establish the effect of radioactive decay within the first generation progeny on the further transferability of the transferred parental isotope.

(a) *Growth of the ^{32}P-labeled parent generation*

A volume of radioactive stock solution containing the desired amount of ^{32}P was evaporated to dryness and resuspended in 0·5 ml. H medium (to which 20 mg/l. tryptophan had been added). The radioactive growth medium was adjusted to neutral pH and inoculated with 10^6 T4 phages/ml. and 5×10^7 B/r bacteria/ml. The infected culture was then incubated for 4 hours at 37°C, after which potassium cyanide to a final concentration of M/100 and one small crystal each of ribonuclease and deoxyribonuclease were added. This lysate was incubated for 10 minutes at 37°C, before it was diluted 4-fold in H medium and centrifuged for 15 minutes at 4000 *g*. Bacteria of the T4-resistant strain B/4 were added to the supernatant fluid to a concentration of 5×10^8/ml. and the centrifugation repeated. The supernatant fluid of the second centrifugation, which contained about 10^9 infective T4 particles/ml. prior to radioactive decay, represents the "clarified" lysate employed for the transfer experiments described in the following.

(b) *Parent to first generation progeny transfer*

A culture of *E. coli*, BB, grown in TB to a density of 2×10^8 cells/ml., was centrifuged and resuspended in half its original volume of TB containing M/500 potassium cyanide, infected at a multiplicity of 0·1 phages per cell with an hour-old "clarified" lysate of T4 phages labeled with ^{32}P at a specific activity of 530 mC/mg (or 830 atoms of ^{32}P per phage), and incubated at 37°C for 6 minutes to allow adsorption of more than 98% of the phages. The infected bacteria were then washed free of unadsorbed phage and non-phage ^{32}P by two low-speed centrifugations and washings in salt-poor TB at room temperatures and finally resuspended at a density of 10^8 cells/ml. in salt-poor TB at 37°C. The radioactivity contained in this resuspension was measured (count I) and represents the amount of ^{32}P introduced into the cells by the parent phages. The infected, washed bacteria were then incubated at 37°C with aeration to permit intracellular phage growth, until 35 minutes later their lysis was induced by M/100 potassium cyanide, yielding an average first generation progeny burst of 150 phages/cell. Unlysed bacteria and bacterial debris were removed from the lysate by two

successive low-speed centrifugations (a thick suspension of strain B/4 was added prior to the second of these centrifugations to provide a carrier for the sedimentation of residual bacterial debris). The final, clarified supernatant fluid containing the first generation progeny was assayed for its titer of infective phage particles. The amount of radiophosphorus contained in the progeny was determined by infecting a suspension of 4×10^8 cells/ml. of strain BB in TB with an aliquot of this clarified lysate at a multiplicity of infection near unity, incubating the mixture at 37°C for 6 min to permit adsorption of more than 98% of the progeny phage, centrifuging the infected bacteria at low speed, and counting of the radioactivity contained in this pellet (count II). The ratio of count II to count I represents the extent of transfer of parental phosphorus to the first generation progeny, which in the two experiments reported in Fig. 1 amounted to 40 and 38% respectively.

An aliquot of the initial suspension of infected bacteria of count I was also assayed for its infectivity before and after shaking with chloroform in order to determine respectively the fraction of the infective centers which survived washing and the fraction of unadsorbed, infective parental phages which may have still been carried over from the adsorption mixture (Frédéricq, 1952; Séchaud & Kellenberger, 1956). These assays showed that in all of the experiments presented here at least 90% of the infective centers survived washing and no more than 3% of unadsorbed infective parents were ever carried over into the final lysate. Controls were also carried out to determine whether any direct assimilation of non-phage ^{32}P by the bacteria contributed to either count I or count II by exposing cultures of B/4 to the "clarified" lysates of parental and progeny T4 populations under conditions identical to those under which phages of these same lysates were allowed to adsorb to cultures of strain BB in the transfer experiment. Measurements of the radioactivity sedimentable with the B/4 bacteria showed that in both cases the uptake of non-phage ^{32}P by the bacteria does not constitute an important fraction of the experimental counts.

(c) *First to second generation progeny transfer*

In order to test the effect of ^{32}P decay on the transferability to the second generation progeny of the parental phosphorus, the clarified lysate of first generation progeny was stored at 4°C, being assayed periodically for its infective titer. Transfer experiments identical to the one described above were then carried out on the very first day before any decay had occurred and repeated on successive days with aliquots of this stored suspension of first generation progeny. For this purpose, bacteria were infected at a multiplicity of 0·1 first generation progeny per cell, and after 6 min adsorption washed free of cyanide, unadsorbed phage and non-phage ^{32}P. The radioactivity adsorbed to the bacteria was counted (count I) and indicates the amount of parental ^{32}P introduced into the cell by the first generation progeny. The infected bacteria were then incubated as before to produce a crop of about 150 second generation progeny per cell. The amount of radiophosphorus contained in the second generation progeny so produced was again determined by counting the radioactivity of a bacterial pellet to which an aliquot of the progeny had been specifically adsorbed (count II), and the extent of transfer of parental phosphorus from first to second generation progeny calculated in each successive experiment from the daily ratios of count II to count I.

The result of two such experiments is presented in Fig. 1, where the percentage of surviving infecting particles of the radioactive parental and the first generation progeny

populations, as well as the percentage of ^{32}P transferred from the first to the second generation progeny, are plotted against the fraction of ^{32}P atoms decayed. It is seen, first of all, that the highly labeled parental phage population was rapidly inactivated with radioactive decay, so that less than 10% of the parental individuals remained alive after the first day. The first generation progeny population, on the other hand, was found to be essentially stable, only a very gradual and slight loss of infectivity having occurred during more than 3 weeks of storage at 4°C. (The slow inactivation of the progeny actually observed must have been due to some noxious factor in the

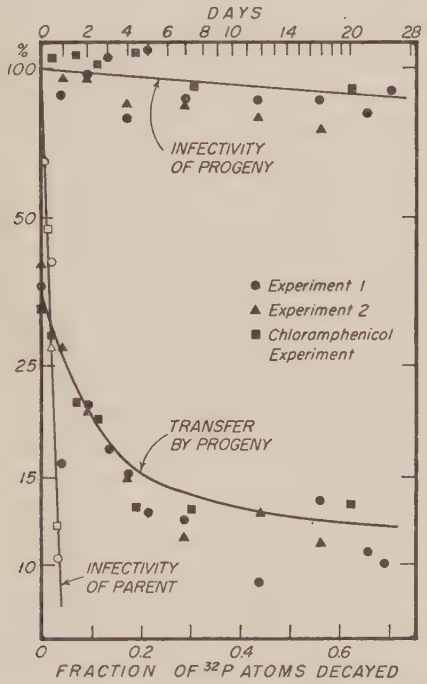

FIG. 1. The percentage of the initial infectivity of highly ^{32}P-labeled T4 parent phages and their first generation progeny, and the percentage of the parental ^{32}P contained in the first generation progeny transferable to the second generation progeny, all as a function of f, the fraction of ^{32}P atoms decayed. (If D is the number of days of decay, then $f = 1 - e^{-0{\cdot}0485D}$). The solid curve drawn through the transfer points has been calculated from equation (4), assuming that 60 % and 40 % of the parental DNA are transferred to the first generation progeny in aggregates of 15 % and 0·3 % respectively of the total DNA complement per parent phage.

lysate, for the progeny populations were perfectly stable if stored at high dilution in fresh medium.) Secondly, it can be seen that on the first day of the experiment, i.e. before any appreciable ^{32}P decay had been allowed to occur in the first generation progeny, 38% of the transferred parental ^{32}P was transferred to the second generation progeny, confirming the fact that the efficiency of the second is similar to that of the first generation transfer (Watson & Maaløe, 1951). On subsequent days, however, progressively decreasing amounts of transfer were found, until minimum transfer values had been attained in the neighborhood of 12% after 0·3 of the ^{32}P atoms had decayed. No further significant decrease in transfer efficiency occurred after that point, approximately 11% of the phosphorus remaining transferable until more than 0·6 of the ^{32}P atoms had decayed.

Control experiments were also carried out with the first generation progeny to assess the reality of the transfer values obtained by the above procedure. For this

purpose, M/100 potassium cyanide was added to an aliquot of the bacteria immediately after their infection with the first generation progeny in order to prevent intracellular phage growth and production of any second generation progeny. This cyanide-inhibited aliquot was otherwise treated exactly as the second generation transfer experiment itself, i.e., incubated for 35 min at 37°C, the "lysate" clarified by two successive low-speed sedimentations, and the radioactivity of the non-existent progeny assayed by adsorption to sensitive bacteria. These cyanide control experiments gave "transfer" values between 2 and 4% showing that the bulk of the ^{32}P measured as having been passed on from parent to progeny generation by the present techniques really appears to reside in progeny phages and was not to be attributed to a small number of highly radioactive parental phages carried over into the progeny population in spite of the repeated low-speed centrifugations and washings.

The data presented in Fig. 1 indicate that the loss of transferability of the parental ^{32}P harbored by the first generation progeny proceeds initially at approximately 10 % of the rate of death of the radioactive parental phage population, in rough agreement with our previous observation (Stent & Jerne, 1955). The specific radioactivity employed in that earlier experiment had, however, not been at a sufficiently high level so that the concave nature of the transfer survival curve of Fig. 1 could be recognized. The final and level part of this curve now reveals that the transferred parental phosphorus is not distributed uniformly among the radioactive individuals of the first progeny generation. Rather, the fact that about 12% of the transferred parental ^{32}P remains transferable even after extensive radioactive decay indicates that a significant fraction of the parental phosphorus is dispersed rather widely among many of the first generation progeny particles. Each of these particles contains so little of the parental radioactivity that decay does not suppress its infectivity at a significant rate. Hence in the process of transfer from parent to progeny, one part of the parental DNA appears to be broken up into small fragments. The rapid loss of transferability of the transferred ^{32}P at the initial stages of radioactive decay indicates, however, that another major fraction of the parental DNA is not so extensively fragmented but instead transferred to a few progeny phages in larger aggregates.

The relative frequency of fragments of various sizes of the transferred parental DNA can be calculated in principle by fitting a curve to the transfer survival data of Fig. 1 and then transforming this curve into a *continuous* distribution function by solving for the coefficients N_i^* and p_i of equation (3). But the experimental points on which this curve would be based are hardly of sufficient accuracy to justify such treatment. Instead, we shall content ourselves with resolving the transfer survival data of Fig. 1 in terms of a two component system, i.e., we shall suppose that a fraction p of the parental ^{32}P atoms is transferred in *large* aggregates of N_1^* atoms each and that the remainder is transferred in *small* aggregates of N_2^* atoms each. Equation (3) then simplifies to

$$T = T_0\left\{p\,\mathrm{e}^{-N_1^*\alpha f} + (1-p)\,\mathrm{e}^{-N_2^*\alpha f}\right\} \tag{4}$$

A fit of (4) to the experimental points, shown in Fig. 1, is obtained if 60% of the parental ^{32}P is assumed to be transferred in large aggregates amounting to 15% of the total ^{32}P content per parent phage and the remaining 40% in small fragments each amounting to only 0·3% of the total ^{32}P per parent.

The finding that the phosphorus, and hence the DNA, atoms of the parent phage are not transferred as a single unit but are instead distributed over many progeny particles could mean that the mechanism of phage DNA replication is *dispersive*,

i.e. that after each reduplication act some of the atoms of the parental molecule are also present in the replica structures. Since a number of successive replication cycles of the phage DNA must take place in order to generate the hereditary substance for several hundred progeny, the parental DNA atoms would become more and more widely dispersed with each such cycle. The fact that some of the parental ^{32}P is passed on in large and some in small fragments would then reflect a non-synchrony of successive replication cycles, i.e. that individual DNA units withdrawn from the vegetative pool for encapsulation into mature progeny particles differ greatly in their proximity of descent from the progenitor phage and hence in their content of parental phosphorus atoms. In other words, the two fractions of the parental phosphorus transferred as large and as small aggregates would represent respectively DNA molecules which have experienced few and many such replication cycles. If this interpretation is correct, then it would be expected that the dispersal of the parental phosphorus among the progeny will be greater if the onset of maturation in phage-infected cells is artificially delayed and additional rounds of DNA replication allowed before the end of the normal eclipse period. Such conditions can be created if protein synthesis in phage-infected cells is inhibited half way through the eclipse period by addition of chloramphenicol to the culture medium (Melechen, 1955; Tomizawa & Sunakawa, 1956). Under these circumstances, synthesis of phage precursor DNA proceeds at nearly the normal rate, while phage maturation is completely arrested. Only after the antibiotic has once more been removed and formation of phage precursor protein been allowed to take place do intact progeny viruses make their intracellular appearance (Hershey & Melechen, 1957).

(d) *Transfer in chloramphenicol*

In order to examine this point, therefore, the following experiment was carried out. A culture of *E. coli*, BB, was infected with an hour-old lysate of T4, labeled with ^{32}P at a specific activity of 500 mc/mg, under conditions identical to those of the parent to first generation progeny transfer experiment described above. The infected culture, washed, as before, in salt-poor TB to remove potassium cyanide, unadsorbed phage and non-phage ^{32}P, was incubated at 37°C for 6 min before chloramphenicol was added to a concentration of 20 μg/ml. The culture was then incubated for 30 further minutes, at which point the infected cells were cooled in ice, centrifuged, and resuspended in fresh salt-poor TB to remove the antibiotic. The resuspended culture was returned to 37°C and incubated for 90 further minutes, when lysis of the infected bacteria was induced by M/100 potassium cyanide, yielding a burst of 170 progeny phages per cell. This lysate was then treated in a manner identical to that of the first generation progeny of the previous experiment and stored at 4°C. Radiochemical analysis of the progeny by adsorption to sensitive bacteria showed that they contained 32% of the parental ^{32}P. From day to day, second generation transfer experiments identical to those described above were carried out with aliquots of this lysate in order to determine the effect of radioactive decay on the transferability of the transferred parental phosphorus.

The result of this experiment is also presented in Fig. 1. It is evident that the prolonged sojourn in chloramphenicol of the phage-infected culture does not appear to have seriously affected the distribution of parental phosphorus atoms among the progeny making their appearance after removal of the antibiotic. For as in the two "normal" transfers presented in the same figure, also in this experiment the transferability of the transferred parental ^{32}P can be seen to fall off with radioactive decay as if

60% of the parental DNA is passed on into a few first generation progeny particles in large aggregates of 15% of the total phage DNA, while only the remaining 40% is dispersed into very much smaller fragments. Hence contrary to the expectations of dispersive DNA replication, no wider distribution of the atoms of the parental phage DNA appears to be produced when a greater number of intrabacterial reduplication cycles is allowed to occur before the onset of maturation of the vegetative DNA into intact progeny particles.

The chloramphenicol transfer experiment does not yet eliminate the possibility that the observed distribution of transferred parental phosphorus reflects some breakage process operating on the parental DNA during its sojourn in the vegetative pool, such as the repetitive chiasma-type of genetic exchange envisioned by Visconti & Delbrück (1953), since this process could itself have been held in abeyance by the presence of the antibiotic. It is, however, possible to make a more general test for the action of random dispersive forces by examining the distribution of transferred parental phosphorus atoms not only among the first *but also among the second generation progeny*. For if a random process were responsible for the observed distribution of the transferred parental atoms among the first generation progeny, then after one additional growth cycle this process would have had added opportunities to disperse the parental DNA further. In other words, the relative frequency and the size of the large aggregate of parental DNA should be less among the second than among the first generation progeny. It is not possible to predict exactly what increased dispersal is to be anticipated in this case without some detailed assumptions concerning the mode of action of these dispersive forces. One may, however, estimate on the basis of a rather crude model† that in case of random dispersal only 36% of the twice-transferred ^{32}P should remain in large aggregates and that the size of these aggregates should be approximately 8% of the weight of DNA per phage particle.

(e) *Parent to first to second to third generation progeny transfer*

In order to test for the action of random dispersive forces on the phage DNA in successive parent to progeny transfers, the following experiment was carried out. A culture of *E. coli*, BB, was infected with an hour-old lysate of T4 labeled with ^{32}P at a specific activity of 550 mc/mg and a parent to first generation progeny transfer experiment carried out as above. One portion of the first generation progeny, containing in this case 31% of the parental ^{32}P, was used immediately for a first to second generation progeny transfer experiment in a manner likewise identical to that already described. The second generation progeny so produced, which contained 15% of the parental ^{32}P or 49% of the ^{32}P possessed by the first generation progeny, were then stored at 4°C. In order to test the effect of ^{32}P decay on the transferability to the *third* generation progeny of the parental phosphorus now already transferred through two successive growth cycles, a transfer experiment again identical to that already described was carried out at once and then repeated on successive days with aliquots

† This model envisions that the observed dispersal of the parental DNA among the first generation progeny arises in two steps: in the course of intracellular growth the entire parental DNA is first cut at random six times to generate large fragments of average size about 14%; of these large fragments a random 40% then suffers many further cuts to generate the multitude of small 0·3% fragments. In the second growth cycle, the once-transferred parental DNA would then be subjected to six further random cuts, generating 8% fragments of which 40% would once more be chosen at random to suffer numerous further cuts. Hence among the second generation progeny, the size of the remaining large fragments of the twice-transferred parental DNA should be 8% and their frequency $60 \times 60\% = 36\%$ of the total.

of the stored suspension of second generation progeny. However, in order to determine the amount of ^{32}P contained in the third generation progeny, whose average specific activity is less than 10^{-4} times that of the original parental generation, a slightly different procedure had to be employed. To an aliquot of the final clarified third generation lysate in salt-free TB, NaCl was added to a concentration of 5 g/l. and a pellet of bacteria harvested from an exponentially growing culture of BR2 resuspended in this aliquot to a density of 5×10^8 cells per ml. Adsorption of the third generation progeny was then allowed to proceed for 6 min at 37°C before the infected bacteria were centrifuged at low speed and the entire pellet transferred to a cup for radioactive counting (count II). A second portion of the first generation progeny was also stored at 4°C and first to second generation progeny transfers made from day to day to ascertain the distribution of parental ^{32}P among the first generation progeny.

The results of this experiment are presented in Fig. 2, where it may be seen, first of all, that before any ^{32}P decay had occurred in the second generation progeny, 42% of the already twice transferred parental ^{32}P was transferable to the

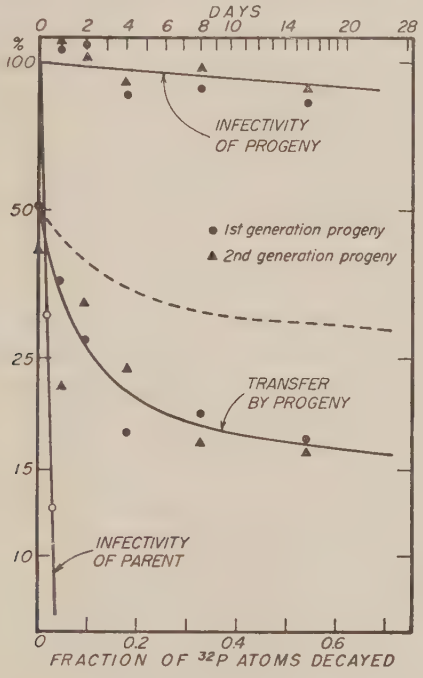

FIG. 2. The percentage of the initial infectivity of highly ^{32}P-labeled T4 parent phages and their first and second generation progeny, and the percentage of the parental ^{32}P contained in the first and second generation progeny transferable respectively to the second and third generation progeny, all as a function of the fraction of ^{32}P atoms decayed. The solid curve drawn through the transfer points has been calculated as in Fig. 1. The broken curve indicates the expected percentage of ^{32}P transferable from second to third generation progeny if the parental DNA were subject to repeated random breakage in successive growth cycles.

third generation progeny, indicating that the efficiency of the third is similar to that of the first and second generation transfers. This reinforces further previous conclusions (Watson & Maaløe, 1951) that the DNA which is transferred neither originates from a preferentially transferable part which is conserved nor is destined for a part which is preferentially lost in the next infection. On subsequent days progressively decreasing transfers from second to third generation progeny were found, until

minimum transfer values in the neighbourhood of 17% had been attained. Comparison of the time course of the transferability of parental ^{32}P from second to third generation progeny shows that radioactive decay affects both of these phage populations in a rather similar manner. For the loss of transferability with decay of the parental ^{32}P harbored by the second generation progeny is also reasonably accounted for by equation (4) if these phages still possess 60% of the transferred parental ^{32}P in aggregates of 15% of the total isotope per parent phage. In contrast, a theoretical transfer survival curve also drawn in Fig. 2, and calculated from (4) for the random dispersal prediction that among the second generation progeny only 36% of the transferred parental ^{32}P is present in aggregates of 8% of the total isotopes per parent phage (and the remainder of the parental ^{32}P in very much smaller fragments), evidently does not fit the experimental data. Hence neither the frequency of large fragments of labeled parental DNA nor their size appears to have been seriously diminished by a second transfer growth cycle, rendering it unlikely that the observed transfer distribution among the first generation progeny resulted from the action of forces causing a random dispersal of the parental DNA in any of the many steps intervening between its injection into the host cell and its encapsulation into a mature progeny virus particle. Instead one may infer that there must exist an *a priori* division of the phage DNA into at least two parts: one part constitutes about 60% of the total and is transferred in fragments of about 15% of the total DNA complement, which fragments are transferred intact through successive growth cycles; the second part constitutes the remaining 40% and is pre-destined to be dispersed over many progeny phages in the course of vegetative phage reproduction.

5. Discussion

The results presented here may now be compared with the distribution of transferred bacteriophage phosphorus determined by Levinthal (1955, 1956) through direct quantitative autoradiographic estimation of the ^{32}P content of individual parental and progeny particles. These experiments showed that approximately half the phosphorus transferred from parent T2 bacteriophages to first generation progeny was found in fragments amounting to $22 \pm 3\%$ of the entire parental DNA, while the remaining half had been transferred in fragments too small to be detected by the radioautographic method. When the first generation T2 progeny were passed through another growth cycle Levinthal observed that the size of the large fragments of labeled parental DNA among the second generation progeny was still $23 \pm 3\%$ of the entire parental DNA and that the frequency of these fragments relative to the total transferred radioactivity had remained unchanged. The direct autoradiographic method thus leads to conclusions analogous to these of the radioactive decay method, in that about half of the parental phage DNA is seen to be widely dispersed in the process of transfer to the progeny and half is less widely dispersed, being transferred in the form of sizable fragments which do not appear to be subject to any further dispersal in a second growth cycle. This evident agreement between the results of the transfer decay method and of autoradiography also appears to justify the assumption made at the outset of our work that the decay of transferred ^{32}P inactivates the T-even particles with an efficiency similar to that of the decay of randomly incorporated radiophosphorus.

The existence of a bipartite nature of the T-even DNA complement finds confirmation from a number of different directions. The autoradiographic method reveals, first of all, that one fraction of the DNA released from the phage head by

osmotic shock appears as a single aggregate containing 36% of the total phosphorus per particle, the remainder evidently being in fragments too small to be detected by this method (Levinthal, 1956; Thomas, 1959). Secondly, the DNA extracted from the phage by chloroform can be resolved by histone column chromatography into two fractions, one 30% and the other 70% of the total, which differ from one another in their purine-pyrimidine composition (Brown & Martin, 1955). Lastly, the T-even phage DNA appears to be composed of two parts which differ in their sensitivity to ultraviolet irradiation and which do not remain associated during parent to progeny transfer (Hershey & Burgi, 1956). It is possible, therefore, that some or all of these fractions correspond to those parts of the phage DNA which differ in respect to the fragment size in which they are transferred. It should be emphasized, however, that it has only been assumed but not actually shown in either the present work or in *any* of the experiments cited above that the bipartiteness of the viral DNA actually obtains within individual phage particles and does not instead reflect some heterogeneity of the phage *population*. Inasmuch as T-even phage stocks can actually be shown to be heterogeneous with respect to their sedimentation in cesium chloride gradients (Pratt & Stent, unpublished observations), some caution might be exercised concerning this assumption.

The finding that the nucleic acid complement of the T-even parental phage is dispersed over a number of its progeny is in perfect harmony with the observation of Tomizawa (1958) that the DNA molecules contained in a single phage particle may have been synthesized at different times of the latent period but that the association of "early" and "late" molecules does not persist through parent to progeny transfer. It appears therefore that the pool of vegetative phage precursor nucleic acid in the infected bacterium is made up of DNA subunits which are assembled into a complete viral nucleic acid complement only upon maturation of the infective particles. Since the parental DNA also forms part of the vegetative pool constituted by its replicas (Hershey, 1953), one may suppose that the observed dispersal of the viral phosphorus atoms in parent to progeny transfer reflects nothing but the dissociation of the parental nucleic acid complement into the subunits from which it had been assembled in the first place in the previous host cell.

One may now enquire whether there corresponds to this dispersal of the molecular patrimony also an analogous dispersal of the *genome* of the parental particles, as would necessarily have to be the case if *all* of the DNA molecules of the T-even phage are really the carriers of a single (or "haploid") set of genetic loci. It is not excluded, however, that the hereditary substance of the phage is contained in only a part of the DNA complement of each particle and that the remainder of the DNA molecules play some "non-genetic" or "physiological" role in the initiation of intracellular phage growth. In particular, it seems possible that the genome of the phage resides in only that portion of the viral DNA which is transferred as a large fragment to the progeny and which appears to remain intact in successive transfers. The notion that there is a single physical structure which carries the entire linkage group of known genetic loci of the T-even bacteriophage finds some support from Garen's (Hershey *et. al.*, 1954) and Hershey & Burgi's (1956) observations that there exists an association between transferred phosphorus and parental host range genotype in successive cycles of mixed growth of T2*h* and T2*h*$^+$ phages and from Levinthal & Thomas's preliminary report (1956) that this association is due to a nearly perfect conjunction of host range genotype with the transferred large fragments of parental DNA. On the other hand, an experiment in which we crossed one highly ^{32}P-labeled T2$hr_1r_7^+m^+$ phage

per bacterium (minority parent) with 10 non-labeled T2$h^+r_1^+r_7m$ phages per bacterium (majority parent) suggested that the minority parental genotype *is* dispersed, i.e. that there is not a single "chromosomic" structure which is transferred intact to the progeny. None of the experiments just mentioned can so far be considered as definitive, however; the transfer studies employing only the host range marker *h* show at most that *one* part of the phage is located in that fraction of the phage DNA which is transferred as a large fragment, whereas the four-factor-cross suffers from the defect that the failure to observe any intact minority parental "chromosome" among the progeny could be due to some aberration of normal phage growth produced by one of the genetic markers employed.

Thus the study of the distribution of the transferred parental atoms has revealed further, hithertofore unsuspected, complexities in the process of intracellular phage growth whose meaning for the mechanism of reproduction of the viral genetic structure should be susceptible to clarification by further experiments.

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Proton Magnetic Resonance Studies on Water in the Presence of Various Macromolecular Substances†‡

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Because of the magnetic properties associated with atomic nuclei, it is possible to measure the absorption of electromagnetic radiation (40–60 Mc/s) by protons when a sample is placed in a strong magnetic field (10,000–15,000 gauss). By keeping the radio-frequency constant and varying the magnetic field an absorption curve having a maximum at the so-called resonance value of the field is obtained. Although the area under the absorption curve is proportional to the number of water protons in the sample, the protons in ice absorb over a very wide range of the magnetic field and, consequently, in a water-ice mixture do not contribute measurably to the area of the narrow water proton signal. Thus, it is possible to use this technique for the detection of the postulated ice-like domains of water in the presence of macromolecules. In solutions containing DNA, hyaluronic acid, collagen, gelatin, or myosin, no change in the area of the absorption curve was observed. This speaks against the existence of ice-like domains. In the presence of DNA, however, considerable broadening of the curve was observed. A similar broadening was observed in the presence of graphite particles. D_2O or NaCl had no effect on the broadening. This effect can be interpreted as the result of magnetic inhomogeneities produced by layers of π-electron systems present both in the DNA molecules and in the graphite particles.

1. Introduction

The work of Jacobson, Anderson & Arnold (1954) on the NMR || of the water protons in a DNA solution suggested that a considerable fraction of the water in such a solution is in a bound, lattice-ordered (ice-like) form. Previous streaming dielectric measurements had already led to a similar conclusion (Jacobson, 1955*a*). In view of the profound biological significance of this idea, we decided to study the NMR of the water proton in various systems containing DNA or other macromolecules, with particular attention to the various factors that affect the NMR signal.

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‡ The 40 Mc/s NMR spectrometer used in these experiments was made available through the courtesy of the Chemistry Department of Harvard University, Cambridge, Massachussetts.

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|| Abbreviations used in this paper:

NMR = nuclear magnetic resonance
TMV = tobacco mosaic virus
T_2 = spin-spin relaxation time
DNA = deoxyribonucleic acid
T_1 = spin-lattice relaxation time

A preliminary report of these findings has been given elsewhere (Bothner-By, Balazs & Gergely, 1958). While this work was in progress Jardetzky & Jardetzky (1957) reported NMR studies on the water protons in 1·9 % TMV solutions, indicating an effect similar to that found by Jacobson *et al.* (1954) in DNA solutions.

2. Methods

Measurements were made on Varian Model V4300B and V4302 high-resolution NMR spectrometers, each equipped with a flux stabilizer, operated at a frequency of 40 Mc/s and 60 Mc/s, respectively. In order to obtain as linear a sweep as possible and to be able to sweep through the absorption lines from both directions, the magnetic field sweep was obtained by driving the usual Varian sweep unit with a Hewlett-Packard Model 202A function generator, producing a triangular output at 0·1 c/s.

The area of the NMR signal was obtained by electrical integration, taking the mean of the two areas obtained on sweeping through the absorption line in both directions. The output voltage of the radio-frequency unit was applied via a 40 MΩ resistance to a 2 μF transmitting condenser of low leakage. The charge on the condenser was read by means of a Keithley Model 200B electrometer, Type VTVM. The time constant of the integrating circuit was about 90 sec.

T_1 of the water H^1 signal was measured in the spectrometer operated at a frequency of 60 Mc/s using the direct method (Andrew, 1955).

The line width of the water H^1 signal was measured directly on the recordings of the spectra at the half-height of the signal. The chart was calibrated by the use of 25 c/s side-bands produced by the audio-modulation of the static field (Arnold & Packard, 1951).

Samples were placed in Pyrex glass tubes with an outside diameter of 5 mm. The inside diameter was checked by comparing areas of pure H_2O signals after the tubes had been filled to the same level. These measurements did not differ by more than 3 %. Measurements were carried out at 16 to 18°C without special temperature control.

3. Materials

DNA was prepared from calf thymus according to the method of Signer & Schwander (1949). One preparation of DNA, which was the same material as that used by Jacobson *et al.* (1954), was obtained through the courtesy of Professor Signer, Berne, Switzerland; the other was prepared by us. The two preparations behaved identically in the NMR experiments.

Collagen gels were made from salt-soluble collagen by heating, as described by Gross (1958). Hyaluronic acid, having a molecular weight of about 3×10^6, was prepared from bovine synovial fluid (Balazs, 1958). Myosin was prepared according to the method of Szent-Györgyi (1951), and was used as a gel after thorough dialysis (to ionic strength less than 0·001). Purified calfskin gelatin† was obtained commercially.

Two graphite samples were used. One, designated as "MM", obtained through the courtesy of the Asbury Graphite Mills, Inc., Asbury, N.J., contained 98 % carbon, < 1 % silicon, 0·5 % aluminum, $< 0{\cdot}5$ % iron impurities and traces of magnesium and potassium. The average particle size was 0·59μ. The other sample, designated as "SP", was a special synthetic graphite for spectroscopic use purchased from the National Carbon Company, Division of Union Carbide and Carbon Corporation, N.Y., N.Y. (SP-2, Lot No. 98 W). Spectroscopic analysis showed a barely visible iron line and somewhat stronger sodium, silver, silicon and magnesium lines.

4. Results

The area under the H_2O proton signals in solutions of various substances was determined in the 40 Mc/s and in the 60 Mc/s instrument with the sample in two different parts of the static magnetic field.

† Distillation Products Industries, Division of Eastman Kodak Company, Rochester, N.Y.

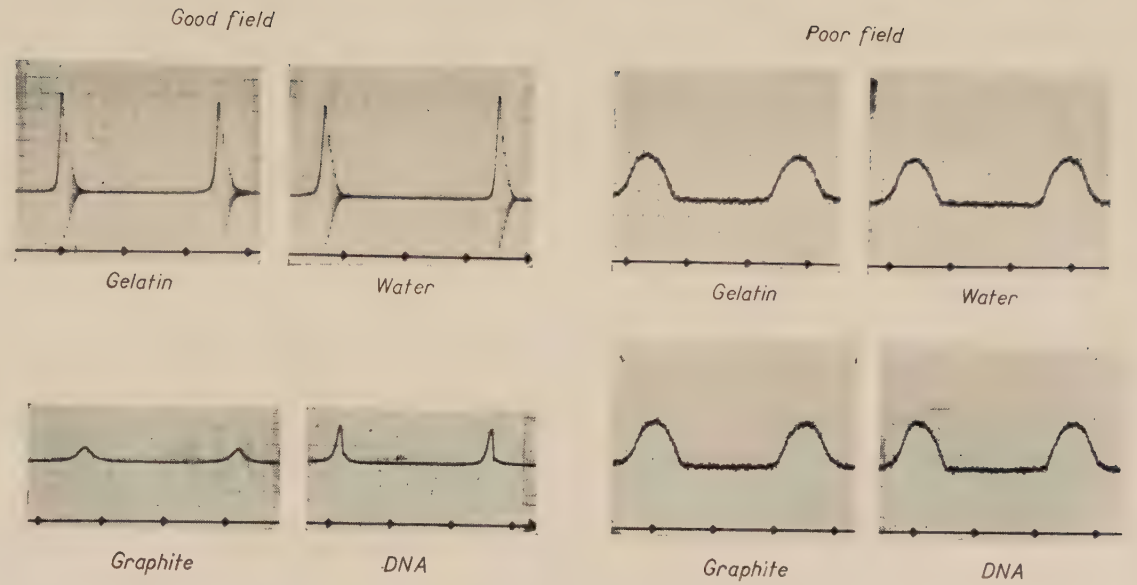

PLATE. 1. Water proton magnetic resonance signals at 40 Mc/s obtained in homogeneous and inhomogeneous magnetic fields. Signals are shown with both increasing and decreasing magnetic field. 5 % gelatin in H_2O, 2 % graphite (MM) suspended in 5 % gelatin gel in H_2O, 2 % DNA in H_2O. Marker along bottom of chart indicates 1 sec intervals.

[*To face page* 148.

The "good field" was characterized by an inhomogeneity, estimated from the line width of the water H^1 signal, of 5×10^{-8}. The "poor field" was characterized by an inhomogeneity of the order of 5×10^{-6}. The intensity of the radio-frequency field was adjusted to a sufficiently low value to avoid saturation, while the leakage voltage was adjusted to a value high enough to avoid distortion of the signal by out-of-phase components of nuclear magnetization. In integrating the signal area, the limits encompassed several line widths on both sides of the signal, thus eliminating errors due to clipping of the wings of the curve. The results of the area measurements are shown in Table 1. It can be seen that DNA and graphite caused a marked decrease

TABLE 1

Area under the water H^1 signal in the presence of various macromolecules and graphite

Sample	Area in arbitrary units with sample in good field	poor field
2 % DNA	0·70	0·64
2 % collagen gel	1·16	0·68
2 % hyaluronic acid	1·22	0·68
5 % gelatin gel	1·20	0·68
2 % myosin gel	1·20	0·66
2 % graphite (MM) in 5 % gelatin gel	0·60	0·66
2 % charcoal in 5 % gelatin gel	1·21	0·66
Solvent (H_2O)	1·24 ± 0·05	0·72 ± 0·04

Measurements made on 40 Mc/s spectrometer.

in the proton signal area in the good field. None of the other substances tested had any significant effect. When the measurements were made in the poor field, the DNA and graphite effect disappeared and all of the water proton signals had the same area. In all of these experiments the field sweep and the radio-frequency field intensity settings of the instrument were kept constant.

The water H^1 signal obtained on the 40 Mc/s instrument in the presence of DNA and graphite in the good field shows a marked broadening in comparison with that of pure water. No broadening was observed with the other substances examined (Plate I). This line broadening explains the area differences observed in the good field, for according to a recent analysis by Williams (1958) of the errors involved in the measurement of integrated NMR signals, spurious area increases occur if the time constant of the resistance-capacitance filter at the cathode of the diode detector exceeds the time needed to traverse the entire signal. If one artificially broadens the line by placing the sample in the poor field, the difference between the signal areas disappears.

Further support for the view that the difference in the area between the DNA and the graphite-containing sample, on the one hand, and the area of pure H_2O on the other hand, is due to an instrumental artifact was obtained from measurements on the 60 Mc/s instrument. This instrument has a phase detection circuit which contains no coupling circuit of long time constant. No area differences were found even in a good field (Fig. 1), but the differences in the line width (Table 2) were identical with those observed on the 40 Mc/s machine.

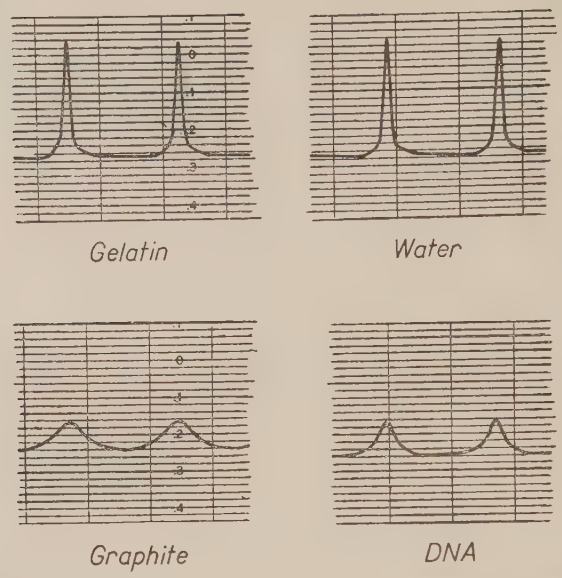

FIG. 1. Water proton magnetic resonance signals at 60 Mc/s obtained in a homogeneous magnetic field. Signals are shown with both increasing and decreasing magnetic field. 5 % gelatin in H_2O, 2 % graphite (MM) suspended in 5 % gelatin gel in H_2O, 2 % DNA in H_2O.

TABLE 2

Effect of various macromolecules and of graphite on the line width of the signal and on T_1 of the water H^1

Sample	Relative line width *	T_1 (sec) †
2 % DNA	2·0	2·1
1 % DNA	1·5	1·9
2 % collagen gel	0·9	2·1
2 % hyaluronic acid	0·9	2·2
5 % gelatin gel	1·0	2·0
2 % myosin gel	1·0	1·9
2 % graphite (SP) in 5 % gelatin gel	4·5	1·9
2 % graphite (MM) in 5 % gelatin gel	4·9	2·0
2 % charcoal in 5 % gelatin gel	1·1	1·9
2 % DNA (0·2 N-NaCl)	1·8	
1 % DNA (0·2 N-NaCl)	1·6	
1 % DNA (50 % H_2O + 50 % D_2O)	1·8	
2 % graphite (MM) in 5 % gelatin gel (50 % H_2O + 50 % D_2O)	5·0	

Measurements made on 60 Mc/s spectrometer.

* The relative line width is the ratio of the line width of the solution and that of the solvent. The solvent was distilled water unless otherwise indicated. The error of these determinations was 10 to 20 %.

† T_1 of distilled water was 1·9. The error of the T_1 determinations was about 20 %.

Measurements of T_1, the so-called "spin-lattice relaxation time", of the water protons revealed no difference between pure water and water in the presence of the substances shown in Table 2.

Jacobson *et al.* (1954) reported that in dilute NaCl solutions the effect of DNA on the water H^1 signal was greatly diminished. We could detect no significant difference between the line width of the water proton signal in DNA solutions with or without NaCl (Table 2).

It seems possible that measurements on solutions in which part of the protons have been replaced by deuterons may cast some light on the mechanism of the broadening. This approach has been applied in the case of TMV by Jardetzky & Jardetzky (1957). As shown in Table 2, the presence of 50 % D_2O as solvent did not significantly change the degree of broadening either in DNA solutions or in graphite suspensions.

5. Discussion

The data reported in this paper show that in aqueous solutions of DNA the water proton signal is broadened. Measurements of T_1 show no appreciable change, nor is the integrated area under the signal different from that obtained from the water signal if instrumental artifacts are eliminated.

Among the macromolecules of biological origin that were tested, DNA was the only substance to cause such broadening. This difference in behavior led us to postulate that a large diamagnetic anisotropy produced by the stacked planar arrangement of purine and pyrimidine bases in the nucleic acid molecule is responsible for the observed effect. In order to test this hypothesis an examination of the behavior of water protons in graphite suspensions was made. Such suspensions showed a behavior quite analogous to and, in fact, more pronounced than that observed in DNA solutions.

The effect observed with DNA and with graphite could not be due to paramagnetic impurities such as iron because no change in T_1 was found and the two graphite samples, only one of which (MM) contained a small iron impurity, produced the same effect.

The observed facts could be interpreted in several ways:

(a) *Hydration shell*

Shaw & Elsken (1953) first reported that the water sorbed by starch and egg albumin (up to 16 % moisture) gives a broadened H^1 signal with the NMR spectrometer. Based on the theory of Bloembergen, Purcell & Pound (1948), this line broadening was interpreted as an increase in the correlation time of the protons in the sorbed water. Jacobson *et al.* (1954) used the same interpretation to explain the apparent decrease in the area under the H^1 signal and the broadening of the line in water containing 1·6 to 60 % DNA. DNA behaved differently from egg albumin, starch and hemocyanin, since it gave a line broadening and an area "decreasing" effect even in relatively low concentration (1·6 %). Based on these results and on streaming dielectric measurements made on water solutions of DNA, Jacobson (1953) suggested that the surface of the two helical polynucleotide chains of the DNA has an ordering effect on the surrounding water lattice. This ordering effect should produce a more regular, ice-like, water lattice around the macromolecule which, in the case of DNA, would extend much further into the solvent than in the case of starch or egg albumin, where the hydration shell presumably extends through only a few molecular layers of water. The biological importance of this concept was stressed by Jacobson (1955*b*) and later elaborated on by Szent-Györgyi (1957) and, more recently, by Klotz (1958).

We have already discussed in the experimental section the factors that may produce an apparent decrease of the area under the NMR signal, and in view of the experiments reported here on both the 40 Mc/s and the 60 Mc/s instrument, it seems safe to rule out an actual decrease of the proton signal area in the presence of DNA or graphite. Thus, at least on the basis of NMR measurements, the position that large domains of water around macromolecules are in an ice-like lattice order, often referred to as "icebergs" (Szent-Györgyi, 1957; Klotz, 1958), seems untenable. The observed broadening of the water signal in the presence of DNA could, however, be interpreted in terms of a rather small hydration shell, as suggested earlier by Onsager (cited by Wang (1955)). The protons in this shell would have a much longer correlation time than the free protons, and consequently, they would have a much shorter nuclear magnetic transverse relaxation time. If one assumes that the rate of molecular exchange between the water in the water phase and that in the hydration shell occurs at high velocity compared with the magnetic relaxation time of the free protons, then broadening of the line would be expected, since the nuclear spins of the protons entering the hydration shell would exchange their energy with those of the bound protons. This mechanism would lead to a decrease in the transverse relaxation time, T_2, the measure of the time during which initially synchronous spins get out of phase. According to this mechanism no change in T_1, characteristic of the exchange of energy between the proton nuclear spins and the lattice, would be expected. This mechanism, then, would account for the observed broadening, for the constancy of the area under the peak, and for the constancy of T_1. However, the fact that no changes whatsoever were observed in the water signal with other polymers in the same concentration forming qualitatively similar gels suggests that if this mechanism is responsible for the changes observed with DNA the hydration of DNA must depend on a special mechanism (see the theory of Jacobson (1953)).

The results of the experiments with D_2O/H_2O mixtures indicate that if this mechanism is the one responsible for broadening in DNA solutions, then the average residence time of a water proton in the hydration shell must be long compared with its transverse relaxation time. If this were not true, the replacement by deuterons of a large proportion of the protons in the hydration shell would increase the probability that a proton could exchange into the shell from the free water phase and then exchange back again without having been magnetically relaxed in the process. DNA would then suffer a loss in efficiency as a broadening agent in these solutions. It is difficult to reconcile this mechanism with the changes observed with graphite, since, in view of its hydrophobic character, the existence of hydration shells is unlikely.

(b) *Magnetic inhomogeneities*

In view of the similarities between the observations on graphite suspensions and on DNA solutions, it occurred to us that the essential feature underlying these observations is not to be sought in a hydration shell but rather in what is common to these otherwise rather different substances. According to the model of Watson & Crick (1953), the DNA molecules consist of double helices with parallel layers of purine and pyrimidine bases containing π-electrons reminiscent of the much more extended layers of the aromatic π-electrons in graphite. Moreover, nucleic acid solutions in the concentration range used in this work possess a partially ordered "liquid crystalline" structure with a hexagonal array of double helices (Riley & Oster, 1951). Structures of this kind should exhibit diamagnetic anisotropy. If one

assumes that the diamagnetic anisotropy of DNA and of graphite plays a significant role in the observed NMR phenomena, two alternative possibilities seem to emerge.

It could be assumed that the diamagnetically anisotropic particles, having rather small translational and rotary diffusion constants, produce long-range inhomogeneities of the magnetic field which, in turn, give the NMR signal in the same way as a static field inhomogeneity does. The condition for such broadening is that during the time interval between energy absorption from the radio-frequency field and energy release to another spin or to the lattice the proton should remain in an essentially homogeneous magnetic field. This condition depends on a proper relationship between the diffusional velocity of the water protons and the size of the magnetic inhomogeneities produced by the particles. In the case of the observations on graphite, gelatin, which alone does not broaden the water resonance line, keeps the graphite particles from settling and also increases the apparent magnetic inhomogeneity by preventing a faster rotation and translation of the graphite particles.

Another possibility is that the regions of magnetic field inhomogeneity caused by the magnetic anisotropy of the DNA molecules or the graphite particles are of such size that the normal diffusional motion of a proton in water could carry it through these regions in times of the order of $1/\nu$, where ν is the Larmor frequency of the proton. The change of the transverse component of the magnetic field in such regions would then cause relaxation by exchange of energy between protons and lattice and thus contribute to line broadening. The condition for most rapid relaxation is:

$$D \approx 2r^2\nu$$

where D is the diffusion constant and r is the mean radius of a region in which the transverse component of the magnetic field is constant in sign. Utilizing the diffusion constant for protons in water at 25°C, as measured by Simpson & Carr (1958), the mean radius, r, for optimum relaxation at 40 Mc/s is calculated to be 50 Å. It would seem quite possible *a priori*, therefore, that this mechanism, in certain cases, could contribute importantly to the total broadening. However, this mechanism would imply a shortening of T_1, which is contrary to the observed results.

It would seem, then, that the line broadening of the H^1 signal should be explained by a mechanism involving the hydration shell and/or large-scale magnetic field inhomogeneities. In the first case, the true transverse relaxation time, T_2, of the protons is expected to change, and this would show up in spin-echo measurements. In the second case, the spin-echo method should yield T_2 values corresponding to those obtained with pure water. Measurements carried out by this method are expected to cast further light on the mechanism of the line broadening of the H^1 signal in DNA solutions and in graphite suspensions.

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The Thermal Denaturation of Collagen in Solution and its Structural Implications

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The thermal denaturation in solution of five purified vertebrate collagens has been examined by viscometry and optical polarimetry. The intrinsic viscosity and specific optical rotation values of undenatured collagen in solution were found to be independent of pH under the conditions used. The temperature dependences of the denaturation processes have been examined for the five collagens as a function of pH and the activation energies and entropy changes involved have been calculated from the changes of intrinsic viscosity with temperature. Considerable and similar variations in the denaturation temperatures T_D with change of pH have been observed for all the collagens; for three collagens this variation is examined in detail. The activation energy involved in the denaturation of a given collagen is found to be independent of pH and the entropy change on denaturation is shown to be a function of the distribution of electric charges along the collagen molecule.

The T_D values for the collagens at a given pH are separately correlated with the contents of hydroxyproline, proline, and total pyrrolidine residues. It is considered that the correlation between T_D and total pyrrolidine content is the most fundamental and that the variation in thermal stability from collagen to collagen may be explained entirely on stereochemical grounds. The results afford no *direct* evidence to allow a decision between the two structures collagen I and collagen II proposed to explain the collagen high-angle X-ray diagram but put both structures on an equal basis. Since collagen II is more stereochemically satisfactory than collagen I this structure would appear to be the more likely.

1. Introduction

Two helical structures for collagen have been published which are consistent with the collagen high-angle X-ray diagram (Ramachandran & Kartha, 1954; Rich & Crick, 1955; Cowan, McGavin & North, 1955). These structures have been termed collagen I and collagen II. Each model is composed of three polypeptide chains (minor-helices) hydrogen-bonded together forming a coiled-coil or major-helix. It has not been found possible to distinguish between the two structures by physical means such as X-ray diffraction or polarised infra-red absorption (Burge, Cowan & McGavin, 1957; Bradbury, Burge, Randall & Wilkinson, 1958). It is a stereochemical requirement of both collagen I and II that every third amino-acid residue along each of the three component minor-helices should be glycine; the restrictions on the disposition of other residues have been examined by Crick & Rich (1957). The amino-acid residue sequence glycine-proline-hydroxyproline can be accommodated along any minor-helix in both structures but in this respect collagen II is much the more satisfactory. In collagen I the hydroxyproline residues are so situated that intra-chain

hydrogen bonds of the OH . . . OC type are possible; in collagen II inter-chain hydrogen bonds of a similar type are possible.

Since a differentiation between the two structures has not been achieved using physical methods (although collagen II is clearly preferable stereochemically) attempts have been made by Doty & Nishihara (1957; Nishihara & Doty, 1958) to resolve the problem by a study of the thermal stability of collagen when in solution, i.e., by examining the conditions under which the hydrogen bonds stabilising the collagen major-helix may be broken. It has been shown by Boedtker & Doty (1955, 1956) that on thermal denaturation the properties of carp swim bladder tunic collagen in solution change from those characteristic of rigid rod-like molecules to those characteristic of randomly coiled single polypeptide chains; these single chains have been identified as the individual collagen minor-helices. Similar results were found by Doty & Nishihara (1957) for calf skin collagen and the concept of a transition of collagen on thermal denaturation in solution from a rod-like molecule to a randomly coiled form is now generally accepted. The thermal denaturation of collagen in solution occurs in a narrow temperature interval (3 to 4°C) and has been observed by Doty & Nishihara for collagens extracted from calf skin, carp swim bladder tunic and cod skin. These authors have found a correlation between the thermal stability and the content of hydroxyproline. This finding appears to indicate that collagen I (which has intra-chain hydrogen bonds involving hydroxyproline) is to be preferred to collagen II.

It seemed desirable to examine further the thermal stability of collagen in solution and in particular to attemj to reconcile the relationship between hydroxyproline content and thermal stability with the stereochemically preferable structure collagen II. Such a reconciliation depends on the expression of the relationship found by Doty & Nishihara in stereochemical rather than chemical terms.

The thermal denaturation of collagen in solution has been examined in this work by means of changes in intrinsic viscosity (Boedtker & Doty, 1956) and specific optical rotation (Robinson, 1953; Cohen, 1955; Boedtker & Doty, 1956) for collagens extracted from rat skin (*Rattus norvegicus*), shark skin (*Lamna nasus*), and the swim bladders of cod (*Gadus morrhua*), perch (*Polynemus quadrifilis*) and maigre (*Sciaena diacanthus*). These collagens were chosen in order to cover as wide a range of hydroxyproline content as possible.

The process of denaturation is conveniently characterised by the "temperature of denaturation" T_D introduced by Boedtker & Doty (1956) and defined as that temperature at which the intrinsic viscosity $[\eta]$ of the collagen has decreased to half its original value. If $[\eta]$ is measured under equilibrium conditions, and it is assumed that the fractional change in the intrinsic viscosity is proportional to the fractional conversion of native into denatured collagen molecules, the temperature dependence of $[\eta]$ may be used to evaluate the activation energy ΔH and the corresponding entropy change ΔS involved in the denaturation process. Since the intrinsic viscosity of denatured collagen is much less than that of native collagen, T_D may be expressed $T_D°\mathrm{K} = \Delta H/\Delta S$.

In this study ΔH and ΔS have been evaluated at two pH values (1·2 and 3·7) for five collagens in an attempt to assess the relative importance of hydrogen bonds and salt links in the stability of the collagen molecule. It is known (e.g., Bowes & Kenten, 1948) that changes in the pH values of collagen solutions do not produce chemical decomposition; the possibility of physical denaturation at low pH values was examined. The thermal stability of the collagens has been studied as a function

of pH and also as a function of the pyrrolidine content of the collagens. An attempt is made on this basis to differentiate between the structures collagen I and collagen II.

2. Experimental

Preparation of solutions and methods of measurement

Collagen extracts ("procollagens") were prepared at 4°C from rat skin, shark skin, and the swim bladders of cod, perch and maigre following a method described by Orekhovich, Toustanovski & Plotnikova (1948). The skins and swim bladders were first extracted with Na_2HPO_4 (0·2 M at pH 9) and the residue extracted with citrate buffer (0·06 M at pH 4·1). The buffer extract was dialysed against 0·01 M-Na_2HPO_4 at pH 8 and the collagen precipitated. The precipitated collagen was filtered and washed and dissolved in a citrate HCl buffer ($\mu = 0{\cdot}075$ at pH 3·7, $\mu = 0{\cdot}063$ at pH 1·2). For one section of the work solutions were made in 0·01 M-acetic acid.

The viscosity determinations were made in viscometers of the Ostwald type at 16°C (water flow time about 250 sec) for all the collagens except that of cod swim bladder which was measured at 4°C. Experiments with multi-gradient viscometers (maximum shear rates in the range 250 to 60 sec^{-1}) showed that under the conditions used the viscosities of all the solutions were independent of the rate of shear. Viscosity measurements were made after aliquots of a given solution had been subjected to an elevated temperature for 30 min (temperature controlled to within $\pm$ 0·01°C) and been quickly cooled to the temperature of measurement (temperature controlled to within $\pm$ 0·1°C). The temperature of measurement was sufficiently low to arrest the denaturation process. Values of the ratio η_{spec}/c where η_{spec} represents the specific viscosity of a collagen solution of concentration c g/100 g of solution, were plotted against c; these graphs were linear within experimental error and were extrapolated to zero concentration to give the intrinsic viscosity values.

The measurements of the specific optical rotations of the solutions for sodium D light $[\alpha]_D$ were made using a Hilger standard polarimeter. The temperature of measurement was controlled to within $\pm$ 0·2°C.

The concentrations of the collagen solutions were determined by the micro-Kjeldahl method after estimations of the total percentage nitrogen content of each collagen (except for shark skin). A nitrogen content of 18·2 % (Eastoe, 1957) was assumed for shark skin.

The proline contents of the collagens were determined by the method of Chinard (1952) as modified by Troll & Lindsley (1955). The contents of hydroxyproline were determined using the method developed by Neuman & Logan (1950). The values for the proline and hydroxyproline content of shark skin were taken from the analysis of Eastoe (1957).

3. The Thermal Denaturation of Collagen in Solution

(a) *Characterisation of undenatured collagen*

The properties of collagen molecules in solutions of different pH before thermal denaturation were examined by viscometry and optical polarimetry. It was found that within experimental error the intrinsic viscosities and specific optical rotation values of solutions in citrate buffer were independent of pH in the range from pH 3·7 to pH 1·2. Values of $[\eta]$ and $[\alpha]_D$ for three collagens at two pH values are given in Table 1. For completeness values of $[\alpha]_D$ for the collagen solutions after thermal denaturation are also included.

The values of $[\eta]$ and $[\alpha]_D$ are very similar to those observed by other workers, e.g., Cohen (1955), M'Ewan & Pratt (1953), Boedtker & Doty (1956), Orekhovich & Shpikiter (1958).

On the basis of the consistency of these physical properties it was concluded that the collagen molecules (except for their states of ionisation) remained essentially physically unchanged when the pH of the solution was varied.

TABLE 1

Variations of the specific optical rotations and the intrinsic viscosities of native collagens with pH

Extracted collagen	Intrinsic viscosity dl.g^{-1}		Specific optical rotation $[\alpha]_D$		$[\alpha]_D$ denatured
	pH 3·7	pH 1·2	pH 3·7	pH 1·2	
Rat skin	13·7 ± 0·2 16°C	13·7 ± 0·5 16°C	− 416° ± 10° 16°C	− 421° ± 8° 16°C	− 133° ± 3° 40°C
Perch swim bladder	13·2 ± 0·5 16°C	15 ± 1 16°C	− 392° ± 8° 16°C	− 370° ± 15° 16°C	− 120° ± 15° 40°C
Cod swim bladder	13·2 ± 0·3 4°C	13·5 ± 0·3 4°C	− 380° ± 4° 4°C	− 382° ± 10° 4°C	− 119° ± 8° 25°C

(b) *Changes in intrinsic viscosity on thermal denaturation*

The variations of the intrinsic viscosities with the temperature t°C at which aliquots of the extracted collagens were heated for 30 min in citrate buffer were determined at pH 3·7 and pH 1·2 for rat skin, shark skin, perch swim bladder, maigre swim bladder

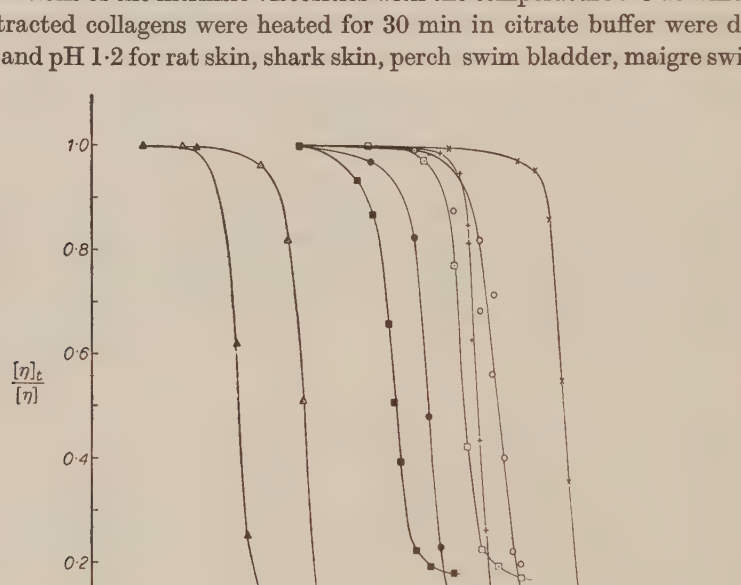

FIG. 1. The ratios of the intrinsic viscosities of aliquots of collagen after heating at t°C for 30 min to the intrinsic viscosities of the native collagens as a function of t°C at two pH values.

Ordinate, $\frac{[\eta]_t}{[\eta]}$: abscissa, temperature t°C.

Key: ×, +, rat skin, respectively pH 3·7 and 1·2, ○, ●, perch swim bladder, respectively pH 3·7 and 1·2, ⊡, ■, shark skin, respectively pH 3·7 and 1·2, △, ▲, cod swim bladder, respectively pH 3·7 and 1·2.

and cod swim bladder. For the purposes of comparison, values of the ratios of the intrinsic viscosities after heating at t°C to the initial intrinsic viscosities of the undenatured solutions are plotted against t in Fig. 1 for four collagens. Curves for maigre are not shown since the variation of $[\eta]$ with temperature for this material was found to be almost identical with that of perch.

The final values of the intrinsic viscosities of the denatured collagens are in the range 0·3 to 0·4 dl.g^{-1} except for shark skin where the value is considerably higher.

The curves in Fig. 1 were assumed to represent the variations with temperature of the concentrations of native and denatured collagen molecules measured at equilibrium and equilibrium constants were calculated. From the temperature dependences of the equilibrium constants, values of ΔH and ΔS, the activation energies and entropy changes appropriate to the processes of denaturation, were calculated. Within the error of measurement ΔH was found to be independent of pH for the five collagens for which it was measured. Mean values of ΔH are given in Table 2 together with values of the denaturation temperatures at pH 3·7 and pH 1·2 and ΔS at pH 3·7. The errors in ΔH have been estimated from the differences between the results of repetitions of the denaturation experiments and the errors in the extrapolations to find the intrinsic viscosities.

TABLE 2

Thermodynamic data characterising the denaturation processes

Extracted collagen	Denaturation temperature °C		Mean ΔH kcal. mol^{-1}	Most probable ΔS cal. mol^{-1} °K^{-1}
	pH 3·7	pH 1·2		
Rat skin	36·5	29·9	340 ± 25	1100
Perch swim bladder	31·3	26·1	200 ± 25	660
Maigre swim bladder	30·0	24·6	200 ± 20	660
Shark skin	28·8	23·5	250 ± 25	830
Cod swim bladder	16·5	11·4	240 ± 25	830

The values of T_D decrease by 5 to 6°C for all the collagens over the pH range considered. The variation of T_D with pH has also been commented on by Orekhovich & Shpikitèr (1957, 1958) who studied extracted rat skin in citrate buffer. The five collagens show a considerable variation in thermal stability at a given pH as judged by the variation (at pH 3·7) of T_D from 36·5°C for rat skin to 16·5°C for cod swim bladder.

(c) *Correlation of denaturation temperature with chemical composition*

Values of the total nitrogen contents and numbers of proline and hydroxyproline residues per 1000 total amino-acid residues (assuming a mean residue weight of 91) are given in Table 3 for the five collagens used in this work.

TABLE 3

Proline and hydroxyproline contents of the collagens

Extracted collagen	Percentage nitrogen	Content of proline per 1000 residues	Content of hydroxyproline per 1000 residues
Rat skin	18·4	130·1	92·6
Perch swim bladder	17·5	118·5	81·2
Maigre swim bladder	17·1	119·1	80·2
Shark skin	18·2 †	113·4 †	78·5 †
Cod swim bladder	18·7	102·7	56·7

† Eastoe (1957)

Values of T_D for these collagens are plotted against the number of hydroxyproline residues per 1000 total residues in Fig. 2 (a) for both pH 3·7 and pH 1·2; the results of Doty & Nishihara (1957) for extracted calf skin, carp swim bladder tunic and cod skin at pH 3·7 are also included. A linear relationship is found. In Figs. 2(b) and 2(c), values of T_D at pH 3·7 are plotted against the total number of pyrrolidine residues

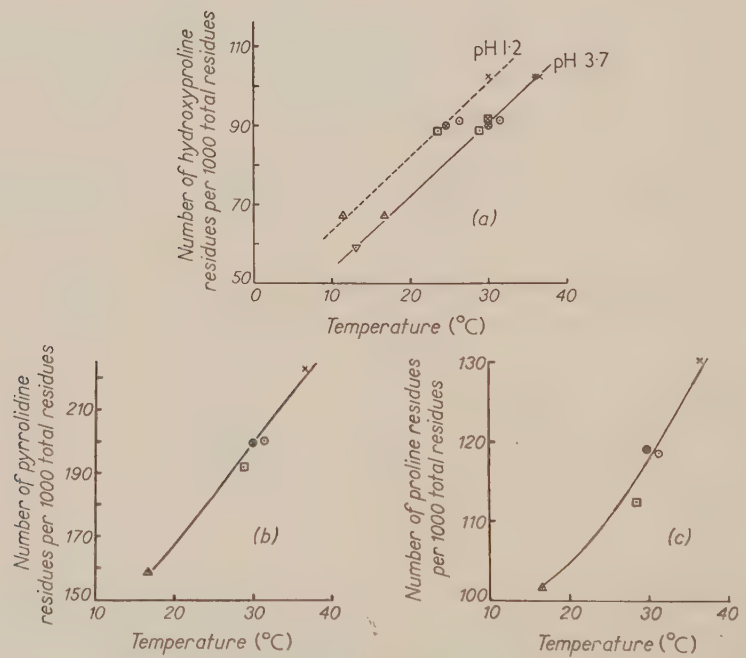

FIG. 2. Variation of denaturation temperature of collagen in solution with content per 1000 total residues of (a) hydroxyproline residues at pH 3·7 and 1·2 (b) total pyrrolidine residues at pH 3·7 (c) proline residues at pH 3·7.

Key: ×, rat skin, ⊙, perch swim bladder, ⊗, maigre swim bladder, ⊡, shark skin, △, cod swim bladder.

Taken from results of Doty & Nishihara (1957), ⁎, calf skin, ⊠, carp swim bladder tunic, ▽, cod skin. Ordinate (a), number of hydroxyproline residues per 1000 total residues. Abscissa (a), temperature °C. Ordinate (b), number of pyrrolidine residues per 1000 total residues. Abscissa (b), temperature °C. Ordinate (c), number of proline residues per 1000 total residues. Abscissa (c), temperature °C.

per 1000 total residues and the number of proline residues per 1000 total residues respectively; the graphs are roughly linear. Similar results were found for pH 1·2 (cf. Tables 2 and 3) but have not been plotted.

(d) *The dependence of the denaturation temperature on pH*

The denaturation temperature is plotted against pH in Fig. 3 for three collagens in acetic acid-HCl solutions. Buffered solutions were found to reduce the value of T_D, e.g., the denaturation temperature of rat skin and cod swim bladder collagens were reduced by 1·5°C and 2·6°C respectively when measured in citrate buffer (for pH 3·7).

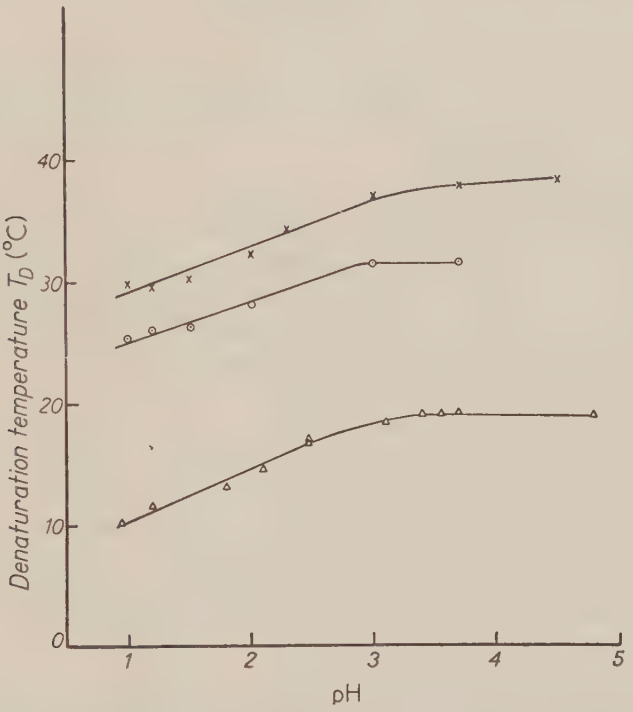

Fig. 3. Variation of the denaturation temperature of collagen in solution with pH.
Key: ×, rat skin, ○, perch swim bladder, △, cod swim bladder. Ordinate: denaturation temperature T_D°C. Abscissa: pH.

The pH values of the solutions were checked immediately before and immediately after each experiment; no significant variation was found. Consistent results for T_D (which was the only result required from this series of experiments) taken from plots of specific viscosity against temperature were found for collagen solutions with concentrations of from 0·1 % to 0·005 % collagen at a given pH.

The thermal stability of the collagens extracted from rat skin and the swim bladders of perch and cod, as judged by the denaturation temperature, is independent of pH in the range from 5 to about 3, at lower pH values the stability decreases considerably. It was shown in the previous section that the activation energy involved in the denaturation process is independent of pH, thus the curves in Fig. 3 essentially represent the variations of $1/\Delta S$ with pH. As the pH decreases below 3 the change in entropy on denaturation increases.

In the range of pH considered, the ionising groups in ox-hide collagen have been identified by Bowes & Kenten (1948) as the carboxylic groups uncomplicated by the

ionisation of other groups. The titration curves given by Bowes & Kenten (1948) may be taken as typical of vertebrate collagens since these have very similar distributions of acidic and basic groups (e.g. Eastoe & Leach, 1957). The titration curve for ox-hide collagen in the absence of salt shows a broad isoelectric region from about pH 10 to pH 3 followed on further decrease of pH by a sharp and roughly linear increase in acid binding by the collagen. It appears that T_D (or $1/\Delta S$) is directly related to the charge distribution along the molecule. In the isoelectric range ΔS is constant and at lower pH values the increase of ΔS is a measure of the increase of the number of positive charges along the molecule.

4. Discussion

It is convenient to divide the discussion into two sections: the first section deals with the process of thermal denaturation of collagen and the properties of the molecule in solution and the second with the problem of differentiating between the structures collagen I and collagen II.

(a) *Thermal denaturation and properties of collagen in solution*

It has been shown that the physical properties of native collagen in solution as judged by optical rotation and intrinsic viscosity measurements are essentially independent of pH. The variation of $[\alpha]_D$ from collagen to collagen is being examined in relation to the correlation between $[\alpha]_D$ and content of pyrrolidine residues pointed out by Szent-Györgyi & Cohen (1957).

The sharp decreases in the values of $[\eta]$ and $[\alpha]_D$ which occur on thermal denaturation are representative of the disruption of the rod-like native collagen molecules to produce separated polypeptide chains of random coil form (Boedtker & Doty, 1956; Doty & Nishihara, 1957). The activation energies and entropy changes involved in the denaturation processes found in this work are of the same order as those which may be calculated for cod skin and calf skin from the results of Doty & Nishihara (1957). The smaller value (81 kcal. mol^{-1}) found by Boedtker & Doty (1956) for carp swim bladder tunic was measured under rather different conditions.

The difficulties of the interpretation of ΔH in denaturation studies have been discussed in detail by Rice & Doty (1957) for the parallel case of the thermal denaturation of deoxyribonucleic acid. Similar difficulties apply to collagen. Obviously ΔH is not a direct measure of the total energy needed to break all the hydrogen bonds stabilising the collagen coiled-coil structure. If a molecular weight of 300,000 (Boedtker & Doty, 1956) is assumed for native collagen and the energy of a peptide hydrogen bond in aqueous solution is taken as 1·5 kcal. mol^{-1} (Schellman, 1955), a ΔH value of about 1500 kcal. mol^{-1} would be expected. If the experimental values of ΔH are *directly* related to the energy changes occurring during the thermal denaturation of the various collagens then the fact that, within experimental error, these values are independent of pH indicates that salt linkages do not contribute significantly to the thermal stability of the collagen major-helix. The values of ΔH for the fish collagens are very similar and considerably less than that found for rat skin collagen. There is no correlation between the ΔH values and the hydroxyproline contents of the collagens.

As the pH of a collagen solution is reduced below the isoelectric range the molecules become progressively more positively charged and the stability of the molecules decreases due to Coulomb repulsions between like charges. The observed decrease of

the denaturation temperature (and so $1/\Delta S$) as the pH of the collagen solutions is reduced below the isoelectric range is therefore readily understood. The similar shapes of the curves of T_D *versus* pH and the similar decreases in the values of T_D on change of pH (Fig. 3) are consequences of the similar numbers of basic and acidic groups found in the various collagens. The variation of T_D at a given pH from collagen to collagen is discussed in the next section.

(b) *The differentiation between collagen I and collagen II*

It is shown in Fig. 2 that correlations may be demonstrated between the denaturation temperature of collagen and the content of hydroxyproline, proline, and total pyrrolidine residues. These three correlations follow essentially from a single correlation and the fact that the ratios of the numbers of hydroxyproline to proline residues are roughly constant in vertebrate collagens. It is difficult to judge which of these correlations is the most fundamental and the correlation chosen by Doty & Nishihara (1957; Nishihara & Doty, 1958) is between T_D and hydroxyproline content which led them to accept collagen I. On this basis a correlation between ΔH and hydroxyproline content might be expected; such a correlation is not observed. It is proposed that the correlation between T_D and the *total* content of pyrrolidine residues be accepted as the fundamental and that the other correlations follow from this.

The correlation between T_D and the total pyrrolidine content may be understood in physical terms by a consideration of the uncoiling of collagen on denaturation. The uncoiling must take place from the ends in order to given rise to the experimentally observed separated, intact, minor-helices. The rigid rods of native collagen have been shown to be about 3000 Å long (Boedtker & Doty, 1956; Hall, 1956) and since the minor-helix repeat distance in either collagen I or II is 85·8 Å then each minor-helix must perform about 35 complete rotations about the major-helix axis to unravel itself. Every third residue along any minor-helix is glycine (within the limits of the models) and the pyrrolidine residues are distributed among the available sites in some way which in the absence of definitive information will be taken to be random. Whenever a pyrrolidine residue is adjacent to another pyrrolidine residue there is a steric hindrance to rotation of one residue with respect to the other. This steric hindrance is demonstrated by the results of experiments on the optical rotations of poly-L-proline and poly-L-hydroxyproline (e.g. Kurtz, Berger & Katchalski, 1956, 1957; Harrington & Sela, 1958). It has been shown by Cowan & McGavin (1955) that poly-L-proline in the solid phase has an exact three-fold left-handed screw axis in the chain direction; the optical rotation results show that this configuration is largely maintained in solution.

It is proposed that the variation in the thermal stability in solution of the various collagens may be explained on the above stereochemical grounds rather than in terms of the hydrogen-bonding capacity of hydroxyproline as proposed by Doty & Nishihara (1957; Nishihara & Doty, 1958). Of course the correlation between T_D and total pyrrolidine content does not in itself distinguish between the models collagen I and collagen II but it shows that *either* model is consistent with the present physicochemical data on collagen solutions. Since of these two models collagen II is the more stereochemically satisfactory it would appear that collagen II is the more likely.

It is not proposed here to discuss the rather different problem of the correlation between the shrinkage temperature of solid collagen samples and hydroxyproline content observed by Takahashi & Tanaka (1953) and Gustavson (1953, 1955).

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The Genetic Control and Cytoplasmic Expression of "Inducibility" in the Synthesis of β-galactosidase by *E. Coli*†

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A number of extremely closely linked mutations have been found to affect the synthesis of β-galactosidase in *E. coli*. Some of these (*z* mutations) are expressed by loss of the capacity to synthesize active enzyme. Others (*i* mutations) allow the enzyme to be synthesized constitutively instead of inducibly as in the wild type. The study of galactosidase synthesis in heteromerozygotes of *E. coli* indicates that the *z* and *i* mutations belong to different cistrons. Moreover the constitutive allele of the *i* cistron is recessive over the inducible allele. The kinetics of expression of the i^+ (inducible) character suggest that the *i* gene controls the synthesis of a specific substance which represses the synthesis of β-galactosidase. The constitutive state results from loss of the capacity to synthesize active repressor.

1. Introduction

Any hypothesis on the mechanism of enzyme induction implies an interpretation of the difference between "inducible" and "constitutive" systems. Conversely, since specific, one-step mutations are known, in some cases, to convert a typical inducible into a fully constitutive system, an analysis of the genetic nature and of the biochemical effects of such a mutation should lead to an interpretation of the control mechanisms involved in induction. This is the subject of the present paper.

It should be recalled that the metabolism of lactose and other β-galactosides by intact *E. coli* requires the sequential participation of two distinct factors:

(1) The galactoside-permease, responsible for allowing the entrance of galactosides into the cell.

(2) The intracellular β-galactosidase, responsible for the hydrolysis of β-galactosides.

Both the permease and the hydrolase are inducible in wild type *E. coli*. Three main types of mutations have been found to affect this sequential system:

(1) $z^+ \rightarrow z^-$: loss of the capacity to synthesize β-galactosidase;

(2) $y^+ \rightarrow y^-$: loss of the capacity to synthesize galactoside-permease;

(3) $i^+ \rightarrow i^-$: conversion from the inducible (i^+) to the constitutive (i^-) state.

The $i^+ \rightarrow i^-$ mutation always affects *both* the permease and the hydrolase. All these mutations are extremely closely linked: so far all independent occurences of each of these types have turned out to be located in the "*Lac*" region of the *E. coli* K 12 chromosome. However, the mutations appear to be *independent* since all the different phenotypes resulting from combinations of the different alleles are observed (Rickenberg, Cohen, Buttin & Monod, 1955; Cohen & Monod, 1957; Cohn, 1957).

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‡ Senior Postdoctoral Fellow of the National Science Foundation (1957–58).

It should also be recalled that conjugation in *E. coli* involves the injection of a chromosome from a ♂ (Hfr) into a ♀ (F^-) cell, and results generally in the formation of an incomplete zygote (merozygote) (Wollman, Jacob & Hayes, 1956). Recombination between ♂ and ♀ chromosome segments does not take place until about 60 to 90 min after injection; moreover segregation of recombinants from heteromerozygotes occurs only after several hours, thus allowing ample time for experimentation.

In order to study the interaction of these factors, their expression in the cytoplasm and their dominance relationships, we have developed a technique which allows one to determine the kinetics of β-galactosidase synthesis in merozygotes of *E. coli*, formed by conjugation of ♂ (Hfr) and ♀ (F^-) cells carrying different alleles of the factors *z*, *y* and *i* (Pardee, Jacob & Monod, 1958). Before discussing the results obtained with this technique, we shall summarize some preliminary observations on the genetic structure of the "*Lac*" region in *E. coli* K 12.

2. Materials and Methods

(a) *Bacterial strains*

A ♂ (Hfr) strain (no. 4,000) of *E. coli* K 12 was used in most experiments. It was derived from strain 58,161 F^+, and was selected for early injection of the "*Lac*" marker (Jacob & Wollman, 1957). This strain is streptomycin sensitive (S^s), requires methionine for growth and carries the phage λ. A second Hfr strain (no. 3,000), isolated by Hayes (1953), was used in some experiments. This strain is S^s, requires vitamin B_1, and does not carry λ prophage. Other Hfr strains carrying mutations for galactosidase (*z*), inducibility-constitutivity (*i*), and permease (*y*) were isolated from the Hayes strain after u.v. irradiation. These markers were also put into ♀ (F^-) strains, by appropriate matings and selection of the desired recombinants.

A synthetic medium (M 63) was commonly used. It contained per liter: 13·6 g KH_2PO_4 2·0 g $(NH_4)_2SO_4$, 0·2 g $MgSO_4 . 7H_2O$, 0·5 mg $FeSO_4 . 7 H_2O$, 2·0 g glycerol, and KOH to make pH 7·0. If amino-acids were required, they were added at a concentration of 10 mg/l. of the L-form. For mating experiments, the above stock medium was adjusted to pH 6·3 and vitamin B_1 (0·5 mg/l.) was added prior to use. Aspartate (0·1 mg/ml.) was generally added at the time of mating, according to Fisher (1957).

(b) *Mating experiments*

The desired volume of fresh medium was inoculated with an overnight culture (grown in the same medium) to an initial density of approximately 2×10^7 bacteria/ml. This culture was aerated by shaking at 37°C in a water bath. Turbidity was measured from time to time; and when the density reached 1 to 2×10^8 bacteria/ml., the experiment was started. Usually small volumes of ♂ and ♀ bacteria were mixed in a large Erlenmeyer flask, with the ♀ strain in excess (e.g. 3 ml. ♂ plus 7 ml. ♀ in a 300 ml. flask). The mixed bacteria were agitated very gently so that the motion of the liquid was barely perceptible. From time to time samples were removed for enzyme assay and plating on selective media, usually lactose-B_1-streptomycin agar, for measurement of recombinants. Under these conditions, in a mating of ♂ z^+Sm^s by ♀ z^-Sm^r, up to 20 % of the ♂ population formed z^+Sm^r recombinants (as tested by selection on lactose-streptomycin agar). More often 5 to 10 % recombinants were found.

Streptomycin (Sm)† was used in many mating experiments, to block enzyme synthesis by z^+Sm^s ♂ cells. Controls showed that the synthesis of β-galactosidase was blocked in these strains immediately upon addition of 1 mg/ml. of Sm. Incorporation of ^{35}S from $^{35}SO_4^=$ as well as increase of turbidity were also suppressed by this treatment. This concentration of Sm had no effect on Sm-resistant (Sm^r) mutants. In some experiments, virulent phage (T6) was used to kill the ♂ cells, thus preventing remating.

† The following abbreviations are used in this paper:

Sm = streptomycin
IPTG = *iso*propyl-thio-β-D-galactoside
ONPG = *o*-nitrophenyl-β-D-galactoside
TMG = methyl-thio-β-D-galactoside

It should be noted that if streptomycin was added initially, it significantly reduced the number of recombinants (e.g., 75 % fewer colonies were formed on lactose-B_1-streptomycin plates after 80 min mating in the presence of 1 mg/ml. streptomycin) relative to mating in the absence of streptomycin; but the antibiotic had little effect on enzyme formation by zygotes if added at the commencement of the experiment or after the z^+ locus had been injected.

When galactosidase synthesis had to be induced in zygotes, *iso*propyl-thio-β-D-galactoside (IPTG) was used at 10^{-3}M, a concentration at which this inducer is known to be active even in the absence of permease (Rickenberg *et al.*, 1956).

(c) *Recombination studies*

The blender technique of Wollman & Jacob (1955) was used to determine the times of penetration of markers into the zygotes. It should be noted that this treatment reduces enzyme-forming capacity in zygotes by 30 to 60 %. Recombinant colonies, selected on appropriate selective media, were restreaked on the selector medium and replica plating was used to determine unselected characters. Tests for galactosidase synthesis (with or without induction) were performed on maltose-synthetic agar plates with or without IPTG, using filter paper impregnated with ONPG, according to Cohen-Bazire & Jolit (1953).

Transductions were performed with phage 363, according to Jacob (1955).

(d) *β-galactosidase assay*

For this enzyme assay, 1 ml. aliquots of culture were pipeted into tubes containing 1 drop of toluene. The tubes were shaken vigorously and were incubated for 30 min at 37°C. They were then brought to 28°C; 0·2 ml. of a solution of M/75 *o*-nitrophenyl-β-D-galactoside in M/4 sodium phosphate (pH 7·0) was added, and the tubes were incubated a measured time, until the desired intensity of color had developed. The reaction was halted by addition of 0·5 ml. of 1 M-Na_2CO_3, and the optical density was measured at 420 mμ with the Beckman spectrophotometer. A correction for turbidity could be made by multiplying the optical density at 550 mμ by 1·65 and subtracting this value from the density at 420 mμ. One unit of enzyme is defined as producing 1 mμ-mole *o*-nitrophenol/minute at 28°C, pH 7·0. The units of enzyme in the sample can be calculated from the fact that 1 mμ-mole/ml. *o*-nitrophenol has an optical density of 0·0075 under the above conditions (using 10 mm light-path).

(e) *Chemicals*

o-nitrophenyl-β-D-galactoside (ONPG), methyl-thio-β-D-galactoside (TMG) and *iso*propyl-thio-β-D-galactoside (IPTG) were synthesized at the Institut Pasteur by Dr. D. Türk. Other chemicals were commercial products.

3. Genetic Structure of the "Lac" Region

Figure 1 presents the structure of the "*Lac*" region, as it can be sketched from the data available at present. This complex locus, as established long ago by Lederberg (1947) and confirmed by the blender experiments of Wollman & Jacob (1955), lies at about equal distances from the classical markers *TL* and *Gal*. The closest known markers are *Proline* (left) and *Adenine* (or *T*6) (right). As shown in the map, the several (about 10) occurrences of the y^- mutation all lie together probably at the left of the segment, while the different z^- mutations and the i^- mutant are packed together at the other end. No attempt has been made to establish the order of individual y^- mutations. The order of the z^- mutations relative to each other and to the i^- marker is unambiguously established, as shown, except for the z_U^- mutation, whose position is largely undetermined. Several independent occurrences of the i^- mutation have been isolated. They all appear to be closely linked to the i_3^- marker, but they have not been mapped, for lack of adequate methods of selection i^+ recombinants. The evidence for this structure is briefly as follows:

(1) The frequency of recombination between z and y mutations is very low:

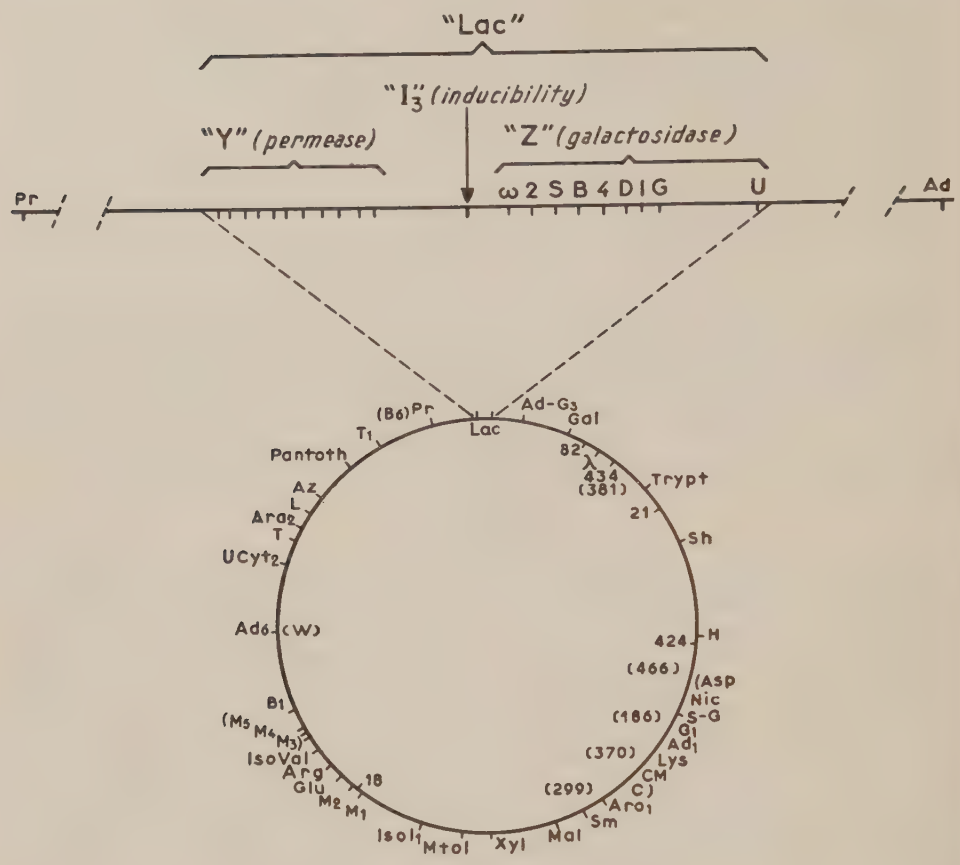


FIG. 1. Fine structure of the "*Lac*" segment.

The "*Lac*" segment is shown enlarged and positioned with respect to the rest of the *E. coli* K 12 linkage group for which the circular model (Jacob & Wollman, 1958) has been adopted.

roughly 1/100th of the frequency of recombination between *T L* and *Gal*. The frequency of recombination between individual *z* markers is about one order of magnitude lower.

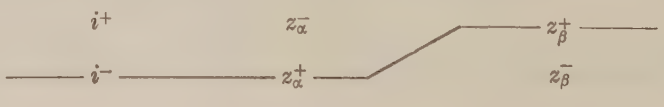
(2) When y^+z^+ recombinants are selected (by growth on lactose-agar) in crosses of the type:

$$y^+i^-z^- \times y^-i^+z^+$$

the i^+ marker remains associated with z^+ 85 % of the time.

(3) The frequency of cotransduction of *i* with *z* (selecting for z^+ alone) is very high (> 90 %), while the frequency for *i* and *y* is also high, although definitely lower (about 70 %). (These data are somewhat ambiguous, because of the heterogeneity of the clones resulting from a transduction.)

(4) The selection of z^+ recombinants in crosses involving different z^- mutants, and *i* as unselected marker, invariably results in about 90 % of the progeny being either i^- or i^+, depending on the particular z^- mutants used. Assuming this result to be due to the position (left or right) of *i* with respect to the *z* group:



a linear order can be established, without contradictions, for the eight markers shown. This however leaves an ambiguity as to whether *i* lies *between* the *y* and the *z* groups, or outside.

Let us emphasize that this sketch of the *Lac* region is preliminary and very incomplete, and that the results concerning the relationships of certain markers are not understood. For instance, the z_U^- marker recombines rather freely with all the other mutants shown (both *y* and *z*) yet, by cotransduction tests, it is closely linked to *i* (25 % cotransduction). It should also be mentioned that certain of the z^- mutants (z_ω^- ; z_2^-; z_G^-) have apparently lost the capacity to synthesize *both* the galactosidase *and* the permease. Yet these mutations do not seem to be deletions. We shall not attempt, here, to interpret this finding, since we shall center our attention on the interaction between the *i* marker and the *z* region.†

A question which should now be considered is whether we may regard the *z* region as possessing the specific structural information concerning the galactosidase molecule. The fact that so far all the independent mutations resulting in loss of the capacity to synthesize galactosidase were located in this region might not constitute sufficient evidence‡. However, it has been found by Perrin, Bussard & Monod (1959, in preparation) that several of the z^- mutants synthesize, instead of active galactosidase, an antigenically identical, or closely allied, protein. Moreover several of these mutant proteins are different from one another by antigenic and other tests. These findings appear to prove that the *z* region indeed corresponds to the "structural" genetic unit for β-galactosidase.

4. β-Galactosidase Synthesis by Heteromerozygotes

(a) *Preliminary experiments*

The feasibility and significance of experiments on the expression and interaction of the *z*, *y* and *i* factors depended primarily on whether *E. coli* merozygotes are physiologically able to synthesize significant amounts of enzyme very soon after mating. It was equally important to determine whether the mating involved any cytoplasmic mixing. These questions were investigated in a series of preliminary experiments.

Since the physical separation of *E. coli* zygotes from unmated or exconjugant parent cells cannot be achieved at present, test conditions must be set up, such that the zygotes only, but not the parents, can synthesize the enzyme. This is obtained when the following mating:

$$♂\ z^+y^+i^+Sm^s \times ♀\ z_s^-y^+i^+Sm^r \qquad \text{(A)}$$

is performed in the presence of inducer (IPTG) and of 1 mg/ml. of streptomycin. The ♀ lack the z^+ factor; the ♂ are inhibited by streptomycin (cf. Methods); the zygotes are not, because they inherit their cytoplasm from the ♀ cells (see below

† Interaction of *i* with the *y* region is of course equally interesting, but since determinations of activity are much less sensitive with the galactoside-permease than with the galactosidase, we have used the latter almost exclusively.

‡ In addition to the mutants shown on Fig. 1, 20 other galactosidase-negative mutants, as yet unmapped, have been found to belong to the same segment by contransduction tests. None was found outside. Lederberg *et al.* (1951), however, have isolated some lactose-"non-fermenting" mutants (as tested on EMB-lactose agar) which are located at other points on the *E. coli* chromosome. In our hands, one of these mutants (Lac_3^-) formed normal amounts of both galactosidase and galactoside-permease (although it did form white colonies on EMB-lactose). Another one (Lac_7^-) formed reduced, but significant, amounts of both. A third (Lac_2^-) which is a galactosidase-negative, appears to belong to the "*Lac*" segment, by cotransduction tests.

pages 170 and 171), and because the type of ♂ used transfers the Sm^s gene to only a very small percentage of the cells. Under these conditions, enzyme is formed in the mated population with a time course and in amounts showing that the synthesis can be due only to zygotes having received the z^+ factor. Figure 2 shows the

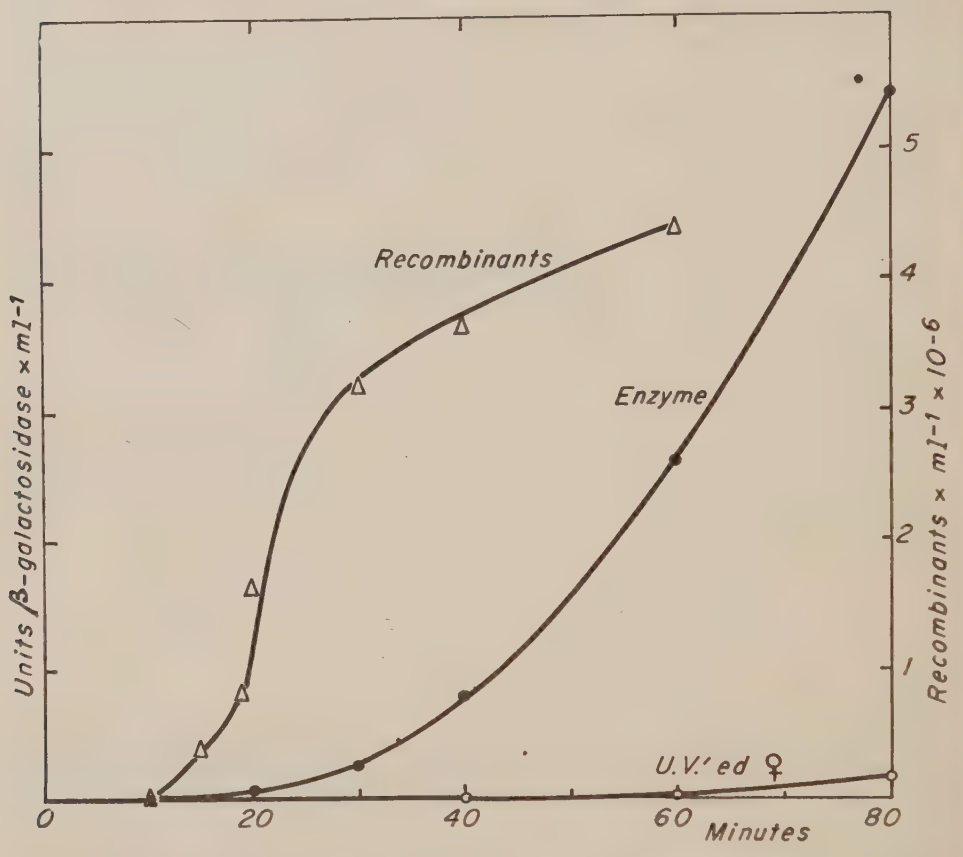


FIG. 2. Enzyme formation and appearance of recombinants in mating A.
Mating in presence of streptomycin (1 mg/ml.) and IPTG (10^{-3}M). A control with u.v.-treated ♀ cells (0·01 % survival) is shown. Recombinants (z^+Sm^r) selected by plating on Sm-lactose agar after blending (separate experiment with the same ♂ culture).

kinetics of galactosidase accumulation, compared with the appearance of z^+Sm^r recombinants, determined on aliquots of the same population (cf. Methods). The latter curve corresponds, as shown by Wollman & Jacob (1955), to the distribution of times of penetration of z^+ genes in the zygote population. It will be remarked that enzyme synthesis commences just within a few minutes after the first z^+ genes enter into zygotes. Assuming that the number of zygotes having received a z^+ gene is 4 to 5 times the number of recovered z^+Sm^r recombinants, and taking into account the fact that normal cells are on the average trinucleate (i.e., have three z^+ genes), the rate of enzyme synthesis per injected z^+ appears nearly normal.

This rapid expression of the z^+ factor poses the problem whether cytoplasmic constituents are injected from the ♂ into the zygote. This already appeared unlikely from the previous observations of Jacob & Wollman (1956). We reasoned that if there occurred any significant cytoplasmic mixing, such a mixing should allow the

♂ cells to feed the ♀ cells with any small metabolites which the ♂ had and the ♀ lacked. This condition is obtained in the following mating:

$$♂\ z^+Sm^s\ maltose^+ \times ♀\ z^-Sm^r\ maltose^-$$

if it is performed in presence of maltose as sole carbon source, using a ♂ which virtually does not inject the *maltose*$^+$ gene. It results in a very strong inhibition of enzyme synthesis (and recombinant formation) showing that the ♂ cannot effectively

TABLE 1

Enzyme formation in nutritionally deficient zygotes

Deficiency	Rate of enzyme formation †			Mean % inhibition of recombinant formation
	Control	Deficient	Mean % inhibition	
Carbon source ‡	1·6	0·4	73	75
	0·66	0·20		
Arginine §	0·28	0·02	96	65
	0·36	0·01		

† Units of enzyme × hr^{-1}.

‡ ♂ z^+Sm^s *maltose*$^+$ × ♀ z^-Sm^r *maltose*$^-$ mated in presence of inducer and Sm, with glycerol plus maltose (control) or maltose as sole carbon source.

§ ♂ z^+Sm^s *Arg*$^+$ × ♀ z^-Sm^r *Arg*$^-$ mated in presence of inducer and Sm with and without arginine (10 μg/ml.).

feed the ♀. An even stronger effect is observed when the ♀ requires arginine, the ♂ not, and mating takes place in absence of arginine (again on condition that the *Ar*$^+$ gene is not injected by the ♂) (Table 1). These observations indicate that even small molecules do not readily pass from the ♂ into the ♀ cell during conjugation.†

It therefore appears that cytoplasmic fusion or mixing does not occur to an extent which might allow cross-feeding. That the contribution of the ♂ is exclusively genetic, and does not involve cytoplasmic constituents of a nature, or in amounts, significant for our purposes, is however only proved by the results of the opposite matings, which we shall consider in the next section.

(b) *Expression and interaction of the alleles of the z and i factors*

We should first consider which of the alleles of the *z* factors are dominant, and whether they all belong to a single cistron. Experiments of the type described above (mating A) were performed with each of the eight z^- mutants, used as ♀ cells, receiving a z^+ from the ♂. Enzyme was synthesized to similar extents in all cases, showing that the z^- mutants in question were all recessive. Each of the mutants was also mated (as ♂) to a z^- ♀. No enzyme was synthesized by any of these double recessive heterozygotes where the mutations were in the *trans* position

† However such leakage may occur when the concentration of a compound is exceptionally high in the ♂. This happens when a ♂ with the constitution $z^-i^-y^+$ is used in the presence of lactose. The constitutive permease then may concentrate lactose up to 20 % of the cells' dry-weight (Cohen & Monod, 1957). Adequate tests have shown that this lactose does flow from the ♂ into a permease-less ♀ during conjugation.

$$\frac{z_\alpha^+ z_\beta^-}{z_\alpha^- z_\beta^+}$$

showing that all the (tested) z^- mutants belong to the same cistron as defined by Benzer (1957).

The next and most critical problem is whether the z and i factors also belong to the same unit of function (gene or cistron) or not. Let us recall that cells with the constitution z^+i^+ synthesize enzyme in presence of inducer only, while z^+i^- cells synthesize enzyme without induction, and z^-i^+ or z^-i^- cells do not synthesize enzyme under any condition. The extremely close linkage of z and i mutations suggests that they may belong to the same unit. If this were so, they would not be able to interact through the cytoplasm, but could act together only when in *cis* position within the same genetic unit. The heterozygote, z^+i^+/z^-i^- would then be expected not to synthesize galactosidase constitutively.

In order to test this expectation, the following mating:

$$\sigma\, z^+i^+ \times \varphi\, z_2^-i_3^- \qquad \text{(B)}$$

was performed *in absence of inducer*. The σ cannot synthesize enzyme, because they are i^+. The φ cannot because they are z^-. The zygotes however do synthesize enzyme (Fig. 3): during the first hour following mating the synthesis is, if anything, even more rapid and vigorous than when both parents are i^+ and inducer is used, as in mating (A).

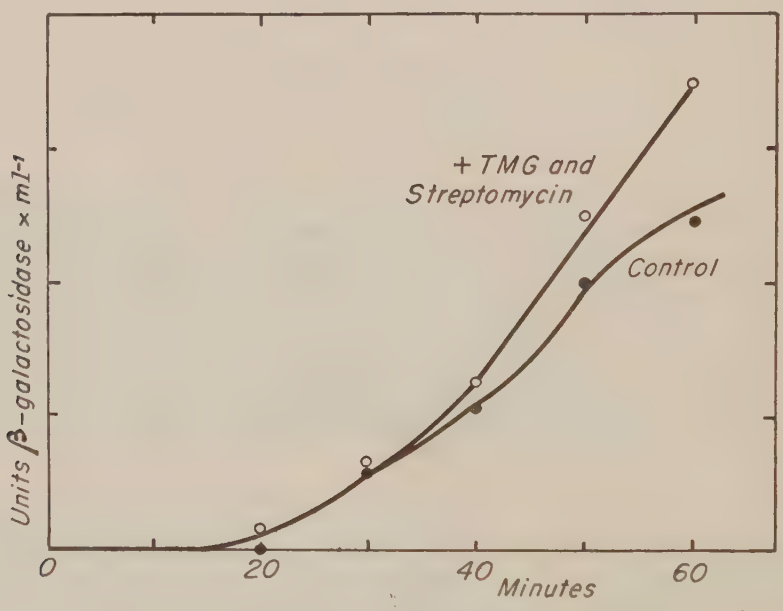


Fig. 3. Enzyme formation during first hour in mating B.
Mating under usual conditions. To an aliquot streptomycin (0·8 mg/ml.) was added at 20 minutes, and TMG at 25 minutes, to allow comparison of synthesis with and without inducer.

Such a mating therefore allows immediate and complete interaction of the z^+ from the σ with the i^- from the φ. The possibility that the interaction depends upon actual recombination yielding z^+i^- in *cis* configuration is excluded because: (a) the synthesis begins virtually immediately after injection whereas genetic recombination is known (Jacob & Wollman, 1958) not to occur until 60 to 90 min after injection; (b) the factors z and i are so closely linked that recombination is an exceedingly rare

event (less than 10^{-4} of the zygotes) while the rate of enzyme synthesis is of an order indicating that most or all of the zygotes participate.

The possibility should also be considered that, rather than taking place through the cytoplasm, the interaction requires actual *pairing* of the homologous chromosome segments. This is excluded by the fact that the following mating:

$$\text{♂ } z^-_2 i^-_3 \times \text{♀ } z^+ i^+ \qquad \text{(C)}$$

when performed in the *absence* of inducer, yields no trace of enzyme, at any time after mixing, although conjugation and chromosome injection occur normally as shown by adequate controls involving other markers. The zygotes obtained in matings B and C are genetically identical, except that the wild type alleles (z^+i^+) are in relative excess (about 3 to 1) in (B), while the mutant alleles are in similar excess in (C). This quantitative difference cannot account for the absolute contrast of the results of the reciprocal matings, one allowing vigorous constitutive synthesis, the other none at all. This can only be attributed to the fact that the cytoplasm of the zygote is entirely furnished by the ♀ cell, with no significant contribution from the ♂. Therefore the $i^- \rightarrow z^+$ interaction must be considered to take place through the cytoplasm.

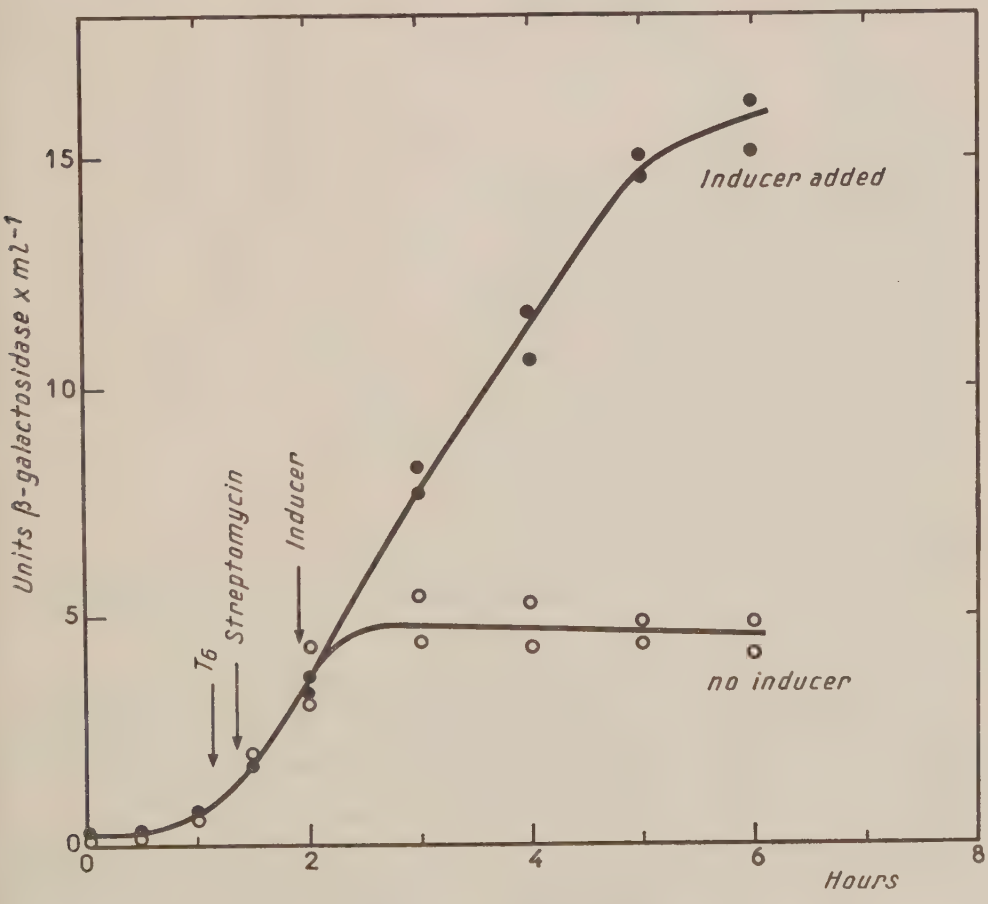


FIG. 4. Enzyme formation in mating D.

Mating performed under usual conditions in quadruplicate in absence of inducer. At times indicated, a suspension of phage T6 (20ϕ/B final concentration) and streptomycin (1 mg/ml.) were added to all of the cultures and TMG (2×10^{-3}M) was added to two of them (black circles) while the other two (white circles) received no addition.

This result may also be expressed by saying that the *i* factor sends out a cytoplasmic message which is picked up by the *z* gene, or gene products. Postulating, as we must, that this message is borne by a specific compound synthesized under the control of the *i* gene, we may further assume that one of the alleles of the *i* gene provokes the synthesis of the message, while the other one is inactive in this respect. If these assumptions are adequate, one of the alleles should be absolutely dominant over the other, but the dominance should become expressed only gradually when the cytoplasm of the zygotes came from the recessive parent, while it should be expressed immediately when the cytoplasm came from the dominant parent.

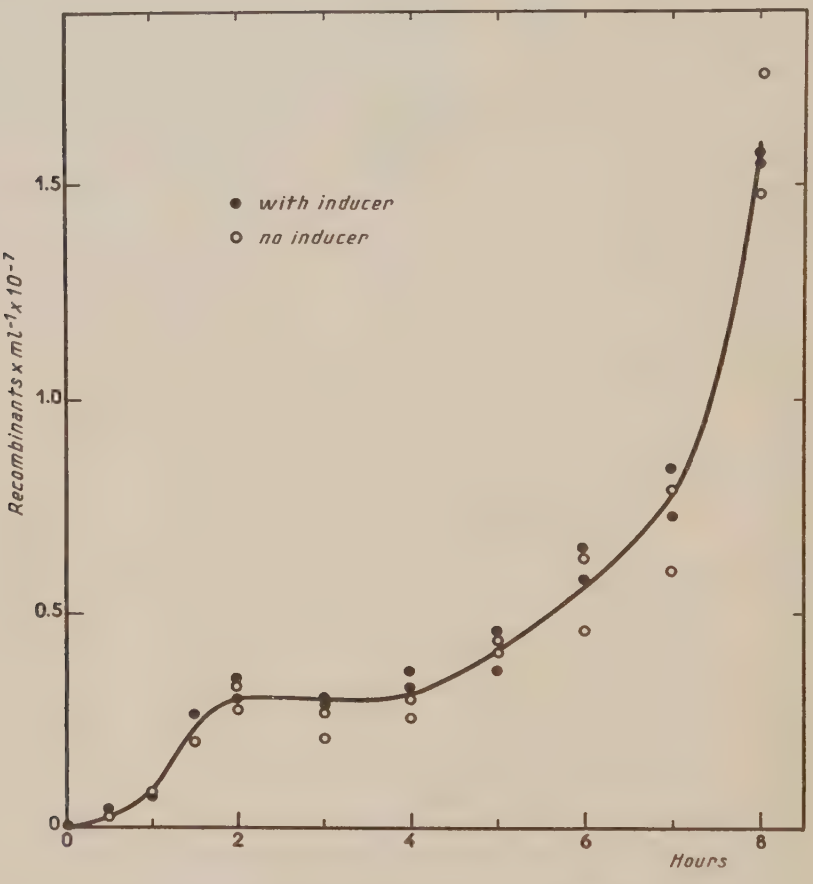


FIG. 5. Recombinant appearance in mating D.

Formation of z^+Sm^r recombinants tested by plating aliquots of the four cultures used in the experiment above (Fig. 4) on lactose-Sm agar. Portions of the culture were diluted 1000-fold and shaken vigorously at 100 minutes to prevent further mating. The increase up to the second hour is due to increasing numbers of zygotes. The increase after the fourth hour is due to *multiplication* of segregants (Wollman, Jacob & Hayes, 1956).

The fact that in matings of type (C) no enzyme is synthesized, even several hours after mating, means that the constitutive (i^-) allele from the ♂ is never expressed. This suggests that the dominant allele is the inducible (i^+). If so, the i^+ should eventually become expressed in matings of type (B)—i.e., the zygotes, initially constitutive (since their cytoplasm comes from the i^- parent), should eventually become inducible. To test this prediction, the following mating was performed:

$$\delta\ z^+i^+Sm^s\ T6^s \times \varphi\ z_2^-i_3^-Sm^r\ T6^r \qquad \text{(D)}$$

and the synthesis of enzyme, in the absence and in the presence of inducer, was followed over several hours (in order to block induction of the ♂ and remating, a mixture of streptomycin and T6 phage was used). Figure 4 shows that, in the absence of inducer, enzyme synthesis stops about 90 min (or earlier) after entry of the z^+i^+ genes into the ♀ cells. When inducer is added at this stage, enzyme synthesis is resumed, showing that the initially constitutive z^+i^+/z^-i^- zygotes have not been inactivated, but have become inducible.

It should be asked whether this conversion to inducibility, rather than occurring in the heterozygotes, might not correspond to the segregation of homozygous $z^+i^+Sm^rT6^r$ recombinants with concomitant disappearance of the heterozygotes. This is excluded because the earliest homozygous recombinants only appear 2 hr after the time when constitutive synthesis ceases† (Fig. 5).

From these observations we may conclude that the constitutive (i^-) allele is inactive, while the i^+ is dominant, provoking the synthesis of a substance responsible specifically for the inducible behaviour of the galactosidase enzyme-forming-center.

5. Discussion and Conclusions

(1) The conclusions which can be directly drawn from the evidence presented above may be summarized as follows:

The synthesis of β-galactosidase and galactoside-permease in *E. coli* is controlled by three extremely closely linked genes (cistrons), *z*, *i* and *y*. The *z* gene determines, in part at least, the structure of the galactosidase protein molecule. The *y* gene probably does the same for the permease molecule, but there is no evidence on this point. The *i* gene in its active form controls the synthesis of a product which, when present in the cytoplasm, prevents the synthesis of β-galactosidase and galactoside-permease, unless inducer is added externally (inducible behaviour). When the *i* gene-product is absent or inactive as a result of mutation within the gene, no external inducer is required for β-galactosidase and galactoside-permease synthesis (constitutive behaviour). The *i* gene product is very highly specific, having no effect on any other known system.

(2) While proving that the interaction of the *i* and *z* factors involves a specific cytoplasmic messenger, the data presented here do not, by themselves, give any indication as to the mode of action of this compound. Two alternative models of this action should be considered.

According to one, which we shall call the "inducer" model, the activity of the galactosidase-forming system‡ requires the presence of an inducer, both in the constitutive and in the inducible organism. Such an inducer (a galactoside) is synthesized by *both* types of organisms. The i^+ gene controls the synthesis of an enzyme which destroys or inactivates the inducer: hence the requirement for external inducer in the wild type. The i^- mutation inactivates the gene (or its product, the enzyme) allowing accumulation of endogenous inducer. This model accounts for the dominance of inducibility over constitutivity, and for the kinetics of conversion of the zygotes.

† It may also be recalled that, according to Anderson & Maze (1957), heterozygosis prevails for many generations in the descendants of *E. coli* zygotes.

‡ By this term we designate the system of all cellular constituents *specifically* involved in galactosidase synthesis. This includes the *z* gene and its cytoplasmic products.

According to the other, or "repressor", model the activity of the galactosidase-forming system is inhibited in the wild type by a specific "repressor" (probably also involving a galactosidic residue) synthesized under the control of the i^+ gene. The inducer is required only in the wild-type as an *antagonist* of the repressor. In the constitutive (i^-), the repressor is not formed, or is inactive, hence the requirement for an inducer disappears. This model accounts equally well for the dominance of i^+ and for the kinetic relationships.

(3) The "repressor" hypothesis might appear strictly *ad hoc* and arbitrary were it not also suggested by other facts which should be briefly recalled. That the synthesis of certain constitutive enzyme systems may be specifically inhibited by certain products (or even substrates) of their action, was first observed in 1953 by Monod & Cohen-Bazire working with constitutive galactosidase (of *E. coli*) (1953*a*) or with tryptophan-synthetase (of *A. aerogenes*) (1953*b*), and by Wijesundera & Woods (1953), and Cohn, Cohen & Monod (1953) independently working with the methionine-synthase complex of *E. coli*. It was suggested at that time that this remarkable inhibitory effect could be due to the displacement of an internally-synthesized inducer, responsible for constitutive synthesis, and it was pointed out that such a mechanism could account, in part at least, for the proper adjustment of cellular syntheses (Cohn & Monod, 1953; Monod, 1955). During the past two or three years, several new examples of this effect have been observed and studied in some detail by Vogel (1957), Yates & Pardee (1957), Gorini & Maas (1957). It now appears to be a general rule, for bacteria, that the formation of sequential enzyme systems involved in the synthesis of essential metabolites is *inhibited* by their end product. The convenient term "repression" was coined by Vogel to distinguish this effect from another, equally general, phenomenon: the control of enzyme *activity* by end products of metabolism.

(4) The facts which demonstrate the existence and wide occurrence of repression effects justify the basic assumptions of the repressor model. They do not allow a choice between the two models. Further considerations make the repressor model appear much more adequate:

(a) The repressor model is simpler since it does not require an independent inducer-synthesizing system.

(b) It predicts that constitutive mutants should, as a rule, synthesize more enzyme than induced wild-type. This appears to be the case for such different systems as galactosidase, amylomaltase (Cohen-Bazire & Jolit, 1953), glucuronidase (Stoeber, 1959, unpublished data), galactokinase of *E. coli* and penicillinase of *B. cereus* (Kogut, Pollock & Tridgell, 1956).

(c) The inducer model, if generalized, implies that internally synthesized inducers (Buttin, unpublished) operate in all constitutive systems. This assumption, first suggested as an interpretation of repression effects, has not been vindicated in recent work on repressible biosynthetic systems (Vogel, 1957; Gorini & Maas, 1957; Yates & Pardee, 1957). In contrast, the synthesis of numerous inducible systems has been known for many years (Dienert, 1900; Stephenson & Yudkin, 1936; Monod, 1942) to be inhibited by glucose and other carbohydrates. The recent work of Neidhardt & Magasanik (1957) has shown this glucose effect to be comparable to a non-specific repression and these authors have suggested that glucose acts as a preferential metabolic source of internally synthesized repressors. If this is so, and if our repressor model is correct, the conversion of glucose into specific galactosidase-repressor should be blocked in the constitutives. Accordingly the galactosidase-forming system of the

mutant should be largely insensitive to the glucose effect while other inducible systems should retain their sensitivity. That this is precisely the case (Cohn & Monod, 1953) is a very strong argument in favor of the repressor model.

(5) If adopted and confirmed with other systems, the repressor model may lead to a generalizable picture of the regulation of protein syntheses; according to this scheme, the basic mechanism common to all protein-synthesizing systems would be inhibition by specific repressors formed under the control of particular genes, and antagonized, in some cases, by inducers. Although the wide occurrence of repression effects is certain, the situation revealed with the present system, namely a genetic "complex" comprising, besides the "structural" genes (*z*, *y*) a repressor-making gene (*i*) whose function is to block or regulate the expression of the neighboring genes is, so far, unique for enzyme systems. But the formal analogy between this situation and that which is known to exist in the control of immunity and zygotic induction of temperate bacteriophage is so complete as to suggest that the basic mechanism might be essentially the same. It should be recalled that according to Jacob & Wollman (1956), when a chromosome from a λ-lysogenic ♂ of *E. coli* is injected into a non-lysogenic ♀, the process of vegetative phage development is started, which involves as an essential, probably as a primary, step the synthesis of specific proteins. When the reverse mating (♂ non-lysogenic × ♀ λ-lysogenic) is performed, zygotic induction does not occur; nor does vegetative phage develop when such zygotes are superinfected with λ particles. The λ-lysogenic cell is therefore immune against manifestations of prophage or phage potentialities, *and the immunity is expressed in the cytoplasm* (Jacob, 1958–59). Moreover the immunity is strictly specific, since it does not extend to other, even closely related, phages. The formation, under the control of a phage gene, of a specific repressor, able to block synthesis of proteins determined by other genes of the phage, would account for these findings.

(6) Implicit in the repressor model are two critical questions, which for lack of evidence we have avoided discussing, but which should be explicitly stated in conclusion. These questions are:

(a) What is the chemical nature of the repressor? Should it be considered a primary or a secondary product of the gene?

(b) Does the repressor act at the level of the gene itself, or at the level of the cytoplasmic gene-product (enzyme-forming system)?

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X-ray Diffraction Studies of the Molecular Structure of Nucleohistone and Chromosomes

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Nucleohistone is probably the basic substance in the chromosomes of higher organisms. The X-ray diffraction patterns of whole nuclei and nucleohistone extracted from them are very similar. Oriented patterns may be obtained from partly-dried fibres of nucleohistone and, although the water content of nuclei *in vivo* is higher than in these fibres, it seems likely that the molecular organisation is much the same. A 35 to 37 Å spacing (not due to DNA) is slightly oriented along the length of the fibres. It is suggested that this spacing corresponds to the distance between labile histone bridges which form links between DNA molecules coiled round each other. The aggregate of coiled molecules forms a coiled fibril. Isolated nuclei in 0·14 M-NaCl solution give a 38 Å reflection and it is suggested that this corresponds to the 35 to 37 Å reflection from fibres. At 98 % relative humidity the characteristic diffraction from DNA molecules is recognisable in the nucleohistone pattern. At low humidity the DNA pattern fades and most of the diffraction is due to consolidated histone. A lipid, resembling sphingomyelin, is associated with the histone in nucleohistone and gives rise to strong diffraction superimposed on the pattern due to nucleohistone itself.

1. Introduction

Nucleohistone appears to be an essential part of the genetic mechanism in higher organisms. DNA in all cells of higher organisms is bound to histone—except in the sperm of some species where histone is replaced by protamine. Although the protein and RNA content of chromosomes in different types of cells varies considerably (Mirsky & Ris, 1951), the relative amounts of DNA and histone do not vary much (Cruft, Mauritzen & Stedman, 1957), and in some types of cells the chromosomes consist largely of nucleohistone. We expect, therefore, that nucleohistone is the basic substance of chromosomes. The X-ray diffraction patterns given by intact nuclei consisting largely of nucleohistone, and by nucleohistone extracted from them, are very similar. We believe that study of these patterns (Wilkins, 1956 *a* & *b*) will reveal the fundamental ultrastructure of chromosomes.

In most of our work we have studied oriented diffraction patterns obtained from fibres of nucleohistone. The orientation gives valuable information and aids interpretation, but the patterns are rather complex and not very sharp and, as a result, their nature, in some respects, is not clear. For instance, it is difficult to decide whether some parts of the patterns are oriented or not, or whether they correspond to one phase of nucleohistone or to different phases. After such matters are decided,

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the further interpretation of the patterns is also ambiguous in some ways. Since we do not expect these difficulties to be overcome soon, we will give now a preliminary account of our results and our interpretation. To link our various observations together, we suggest a general scheme for the macromolecular organisation of nucleohistone. Much of this is speculative.

We have been encouraged to write this paper by reading the MS. by Luzzati & Nicolaieff (1959). Their discussion of nucleohistone as a multi-phase system and a three-dimensional network stimulated our consideration of these matters.

2. Experimental

Calf thymocytes were isolated in 0·14 M-NaCl by chopping tissue and filtering. Fowl erythrocyte nuclei in 0·14 M-NaCl were obtained by laking the cells in water (Stedman & Stedman, 1951). Nucleohistone fragments were prepared by blending calf thymocytes and repeated washing in a solution of 0·075 M-sodium versenate and 0·024 M-NaCl at pH 8 (Zubay & Doty, 1959). Sometimes nucleohistone was purified by dispersing it in water and then collecting it either by sedimenting in the ultracentrifuge, by dialyzing and freeze-drying, or by precipitating in 0·14 M-NaCl. Reconstituted nucleohistone was prepared by mixing solutions of purified histone and DNA in 2·6 M-NaCl and precipitating by adding water.

Wet preparations were X-rayed in stainless steel cells with mica windows. Fibres were equilibrated in a hydrogen atmosphere of controlled humidity in the X-ray camera.

3. Results

(a) *Nucleohistone in a state approximating to that* in vivo

Erythrocyte nuclei or chromosome fragments from thymus in 0·14 M-NaCl give a diffraction pattern the main feature of which is a 38 Å ring (Wilkins, 1956*b*). Other rings at 8, 12, 16, 21, and 28 Å are visible (Plates I*a* and III*b*). There is strong continuous diffraction at angles corresponding to spacings of 60 Å and above. Similar patterns are observed with gels of concentration about 20 % (we will give all concentrations as g of nucleohistone per 100 g of gel). Intact calf thymocyte cells in 0·14 M-NaCl also give the 38 Å ring. We have stretched nucleohistone gels into fibres but, although these are birefringent, we fail to observe orientation of the pattern unless the specimen is dried somewhat. At 92 and 75 % relative humidity the specimens contain respectively 30 and 17 % water.

Plate I. The scale shows distances, measured from the centre of the photographs, corresponding (inversely) to various spacings. Except for Plate III (c), all Plates are on the same scale.

(a) X-ray diffraction pattern of fowl erythrocyte nuclei in 0·14 M-NaCl. The sharp spots are diffraction from the mica windows of the cell. The 8 Å ring is near the edge of the photograph. The 38 Å ring is nearest the centre. This ring and continuous scatter near the centre are shown more clearly on the same scale in Plate III (b).

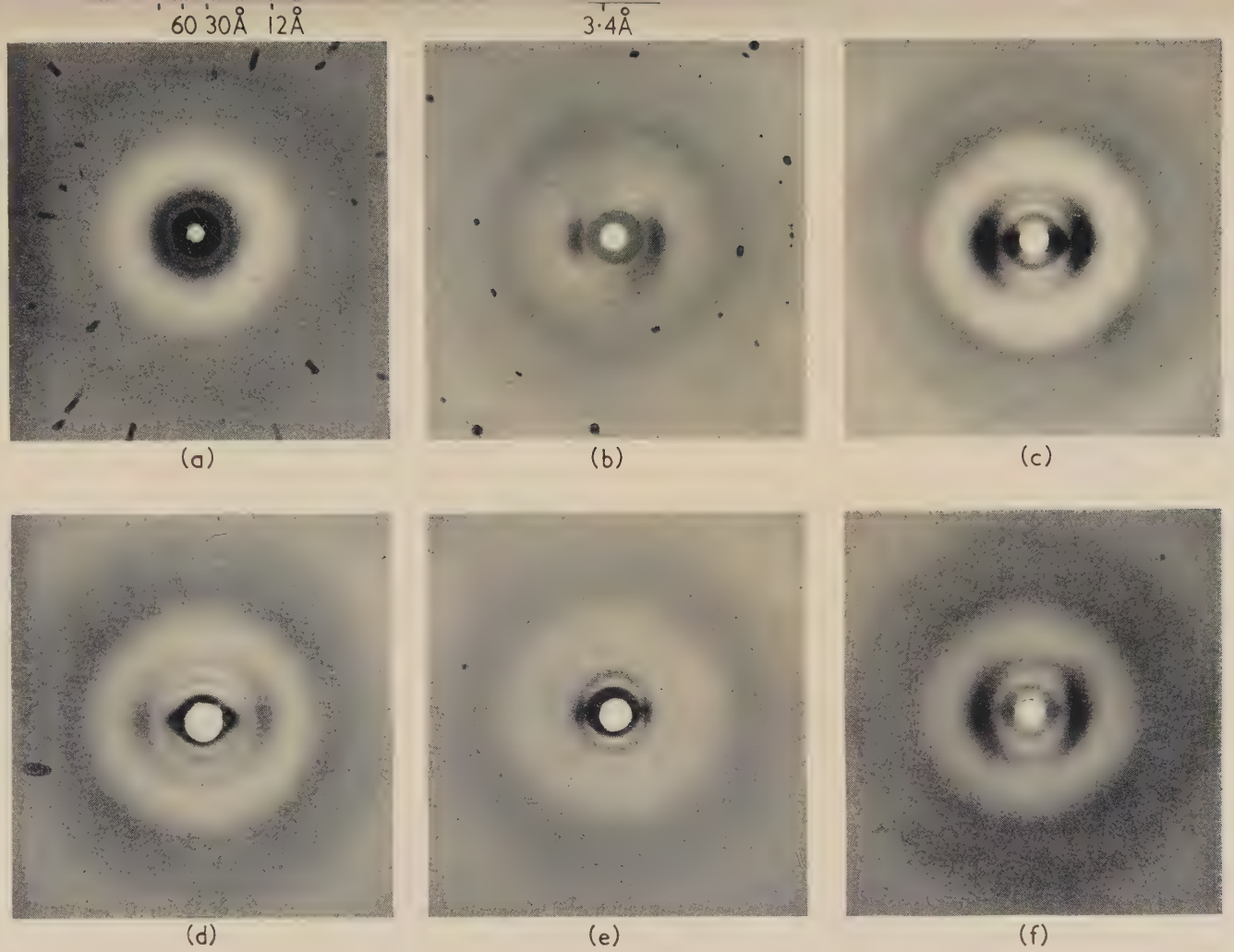
(b) Fibre pattern of nucleohistone fibre, swollen by adding water, and sealed in mica cell (the spots are due to mica). The equatorial spacing is 34 Å. A 37 Å ring is present and the 12 Å ring shows signs of concentration into four regions.

(c) Nucleohistone fibre at 98 % relative humidity. Strong equatorial spots at 30 and 60 Å are visible. Arcs at 35 and 55 Å show traces of meridional orientation. The 8 and 12 Å rings are divided into four sectors.

(d) Nucleohistone fibre at 75 % relative humidity. The equatorial spacing is 22 Å. The orientation of the 35 Å meridional arc can be seen. Rings at 8 and 11 Å are more blurred than the corresponding rings at higher humidities.

(e) Nucleohistone fibre at 32 % relative humidity. Sharp equatorial reflection at 40 Å shows clearly. The poorly oriented meridional arc has spacing of 37 Å. There is a diffuse ring at about 9·7 Å.

(f) Fibre of reconstituted nucleohistone at 98 % relative humidity. All the features of (c) show except that the 60 Å equatorial spot is barely visible.



[*To face page* 180.

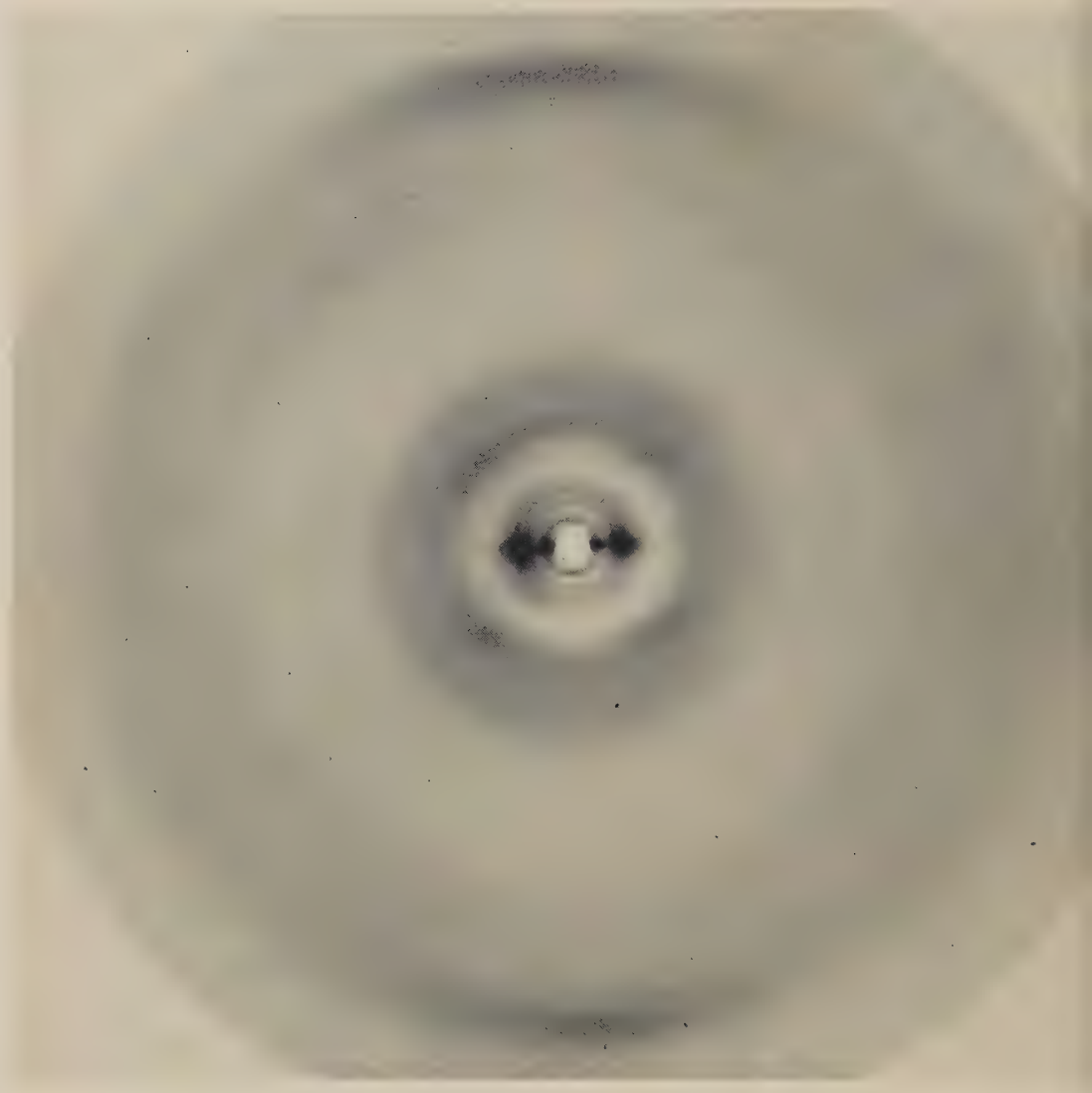


PLATE II. The best oriented pattern we have obtained from a fibre of nucleohistone. Relative humidity is 98 %. The 3·4 Å meridional arc and the first, second and third layer lines of the DNA-type pattern show clearly. The streaks in the outer part of the pattern are very similar to those obtained from DNA at high humidity. The other features are similar to those in Plate I (c).

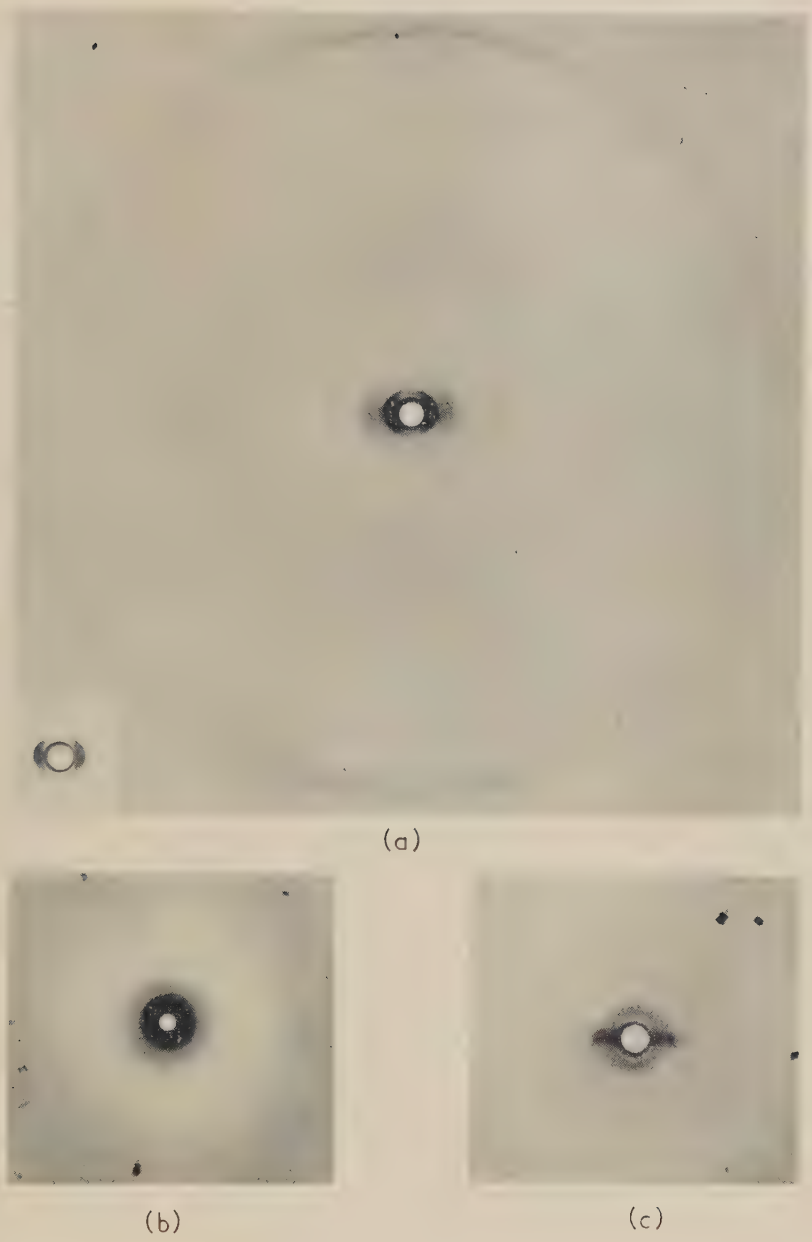


PLATE III (a) Fibre pattern from oriented sphingomyelin. The inset shows the 60 Å equatorial spacing clearly. The 4·2 Å meridional arc and 15 Å and other equatorial spacings may be seen.

(b) Less exposed print of Plate I (a).

(c) Nucleohistone fibre at 75 % relative humidity. The X-ray exposure was less than for Plate I(d) and, as a result, the 75 Å meridional arc can be seen more clearly. The print is 1·5 times the size of those in Plate I.

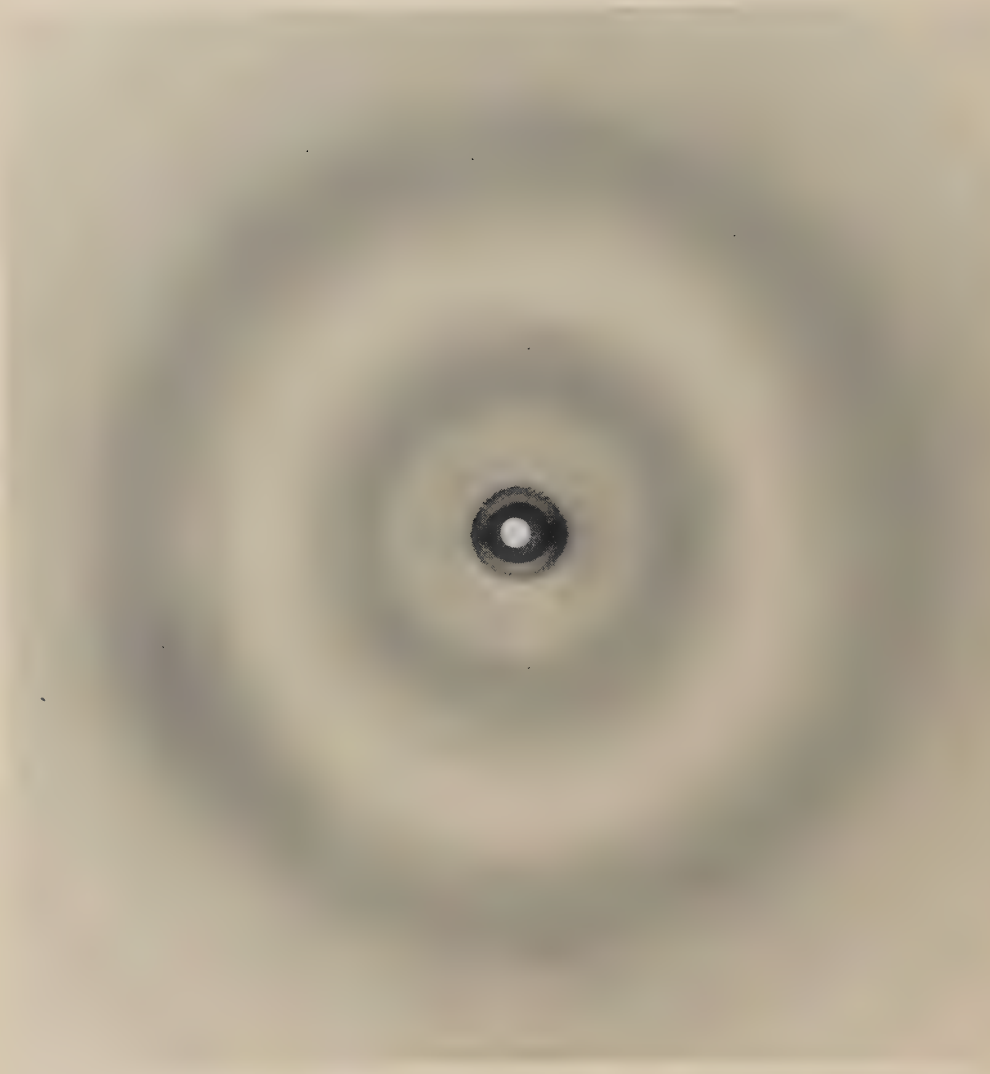


PLATE IV. Nucleohistone fibre in dry hydrogen. The semi-meridional arc has a spacing of 37 Å. The equatorial arc at 35 Å is sharper. There are diffuse unoriented rings at 4·7 and 9·7 Å. Sharp meridional arcs at 4·2 Å show faintly and traces of a diffuse 3·4 Å meridional arc may be seen. Central blackening is intense.

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(b) *Partly-dried nucleohistone*

The diffraction patterns change reversibly when the water content of nucleohistone is varied. Specimens partly dried by equilibrating at 98 % relative humidity give less background scatter than when wet and the pattern shows more clearly. The 38 Å ring is replaced by one at 35 Å and rings appear at 30 and 60 Å. When the material is stretched into fibres the intensity in the rings orients and is concentrated into arcs (Plate II). This pattern appears to consist of three parts.

(a) Much of the pattern resembles that produced by DNA. Near the centre there is an equatorial reflection corresponding to a spacing in the region of 30 Å, and the first, second and third layer lines with layer-line spacing of about 35 Å may be seen. An ill-defined spot is visible on the second layer-line. Densitometry of these layer-lines has so far given no unambiguous indication that nucleohistone molecules diffract differently from DNA molecules. The measurements have limited meaning because we have not identified for certain the crystalline arrangement of the nucleohistone. In the outer part of the pattern there is a 3·4 Å meridional arc and diffuse streaks similar to those given by DNA fibres at high humidity. The best oriented patterns give the impression of consisting of two parts—well oriented streaks along layer-lines and poorly oriented but well defined arcs.

(b) There is a diffraction ring at about 35 Å. When not obscured by equatorial diffraction (Plate I*d*), this reflection may be seen to be poorly oriented in a meridional direction. A ring at about 55 Å shows slight signs of similar orientation (Plate I*c*).

(c) There is a strong well-oriented spot on the equator at about 60 Å. When sheet specimens are photographed parallel to the X-ray beam, this spot is strong and a 15 Å spot may also be seen. When sheets are perpendicular to the beam, both reflections are absent. Often there is a sharp 4·2 Å arc on the meridian (Plate IV). Traces of an equatorial reflection at about 120 Å are sometimes seen on photographs (not shown here) taken with cameras of high resolution.

Oriented specimens of sphingomyelin, about the same size as the nucleohistone specimens, give 60, 15 and 4·2 Å arcs similar to those from nucleohistone but require only about 3 % of the exposure time necessary for nucleohistone (Plate III*a*). The pattern given by reconstituted nucleohistone (made as described above) shows all the features of the nucleohistone pattern except that the 60 Å spot and the 4·2 Å arc are absent. The 35 and 55 Å spacings are visible (Plate I*f*).

The DNA-like pattern is visible over a range of water contents. The spacings corresponding to the spots on the equator and second layer-line both increase with water content. The equatorial spot is visible between 22 and 35 Å (Plate I *b*,*c*,*d*,*e*). At 35 Å it is rather weak and diffuse and, if the water content is increased, a spot is not observed and an unoriented 37 Å ring alone is visible in that region of the pattern.

On drying, the DNA-like pattern becomes diffuse. At 32 % relative humidity and below there are diffuse unoriented rings at 4·7 and 9·7 Å (Plate IV). The 35 Å arc shows clearly and the spacing often increases to about 37 Å. A strong diffuse arc at about 75 Å appears poorly oriented on the meridian and superimposed on continuous scatter (Plate III*c*). At about 75 % relative humidity a sharp equatorial spot often appears at about 45 Å and on drying moves to a limiting value of 35 Å (Plates I*e* and IV).

4. Interpretation of the Diffraction Patterns

(a) *Diffraction from phospholipid impurity in nucleohistone*

We have been reluctant to accept the idea that a small amount of lipid impurity in nucleohistone is responsible for the strong 60 Å reflection characteristic of nucleohistone preparations irrespective of the source from which they are obtained. However, experiment shows that about 3 % of the phospholipid sphingomyelin would give the observed intensities of the 60, 15 and 4·2 Å reflections (see also Schmitt & Palmer, 1940) and the orientation of reflections observed in sheet specimens of nucleohistone. It is difficult to separate phospholipid from nucleohistone. We have observed a 60 Å spacing in some histone preparations but never in DNA. This suggests the lipid is bound to the histone. Chemical evidence supports the idea that nucleohistone has lipid impurity (e.g. Bakay, Kolb & Toennies, 1955) and that the lipid resembles sphingomyelin (Chayen & Gahan, 1958). The sharp 45 Å equatorial spacing that moves to about 35 Å on drying appears to correspond to a shrunken state of the 60 Å sheet structure. This suggests the presence of lipid because a similar reversible shrinkage of sheet-structure spacing from 60 to 40 Å (observed when the temperature is raised to 100°C) (Elkes & Finean, 1953) is characteristic of phospholipids.

(b) *Diffraction from nucleohistone*

(i) *The fibre pattern.* The resemblance of a large part of the pattern to that produced by DNA, shows that DNA in nucleohistone has much the same structure as isolated DNA and that the DNA molecules are roughly parallel to the length of the fibre. The presence of spots on the equator and second layer line indicates that the molecules are packed parallel with some regularity in three dimensions.

The relative intensities of the layer lines of the DNA-type pattern show no very obvious differences from those of DNA itself. This shows more clearly in recent photographs (e.g. Plate II) than in photographs (Wilkins, 1956*b*) obtained earlier. However, the X-ray data are not very accurate and so far we have not been able to decide how histone is attached to individual DNA molecules. Further study of this question is in progress; at the moment it appears that our model involving α-helical histone (Wilkins, Zubay & Wilson, 1959) is more likely to be correct than the earlier model with histone in the β-configuration (Wilkins, 1956*a*).

The 35 Å semi-meridional arc corresponds to a spacing along the length of the fibres and is, therefore, probably along the length of the DNA molecules. The value of the spacing is about the same as the pitch of the DNA helix. A similar meridional reflection is not observed in patterns from DNA, although a streak on the first layer-line sometimes extends across the meridian (e.g. Feughelman *et al.*, 1955, Fig. IV*a*). We suggest that the 35 Å arc is produced by diffraction from histone bridges formed between roughly parallel DNA molecules (Wilkins, Zubay & Wilson, 1959). The properties of nucleohistone in solution suggests the existence of labile bridges. Nucleohistone in water disperses into single molecules of DNA combined with histone (Zubay & Doty, 1959). These nucleohistone molecules aggregate markedly at concentrations above 0·1 %. Since DNA does not aggregate in this way it is clear that histone must form some kind of link between the DNA molecules. We would expect bridges to form between DNA molecules at equivalent positions along the line of junction between two molecules and, therefore, to be spaced about one pitch length apart.

The spacing corresponding to the meridional arc is, at various hydrations, on the average somewhat greater than the observed value for the pitch of the DNA helix. This could be explained as follows. Let the bridge-attachment points on the DNA molecule be spaced apart along the right-handed helix a little more than one turn of the helix, e.g. 11 nucleotides apart instead of 10. The linear separation of the bridge points is then about 38 Å. If two DNA molecules are bent slightly and coiled round each other in a right-handed direction, the bridge points on one molecule come into register with those on the other. Bridges may then form about 38 Å apart. The fitting together of coiled-coil α-helix polypeptide chains is geometrically similar, but in that case (e.g. Crick, 1953) a left-handed helix is coiled in a right-handed manner and a spacing less than the pitch length is produced. The difference between the inter-bridge distance and the usual pitch-length of DNA may not be due to coiling alone: stretching of the DNA molecules (Wilkins, Gosling & Seeds, 1951) may also contribute to the difference.

The 35 Å arc is less well oriented than the main part of the DNA-type pattern, and the DNA-type pattern itself consists of well oriented and poorly oriented parts. This indicates that there are two phases in the fibres—one well oriented and the other poorly oriented. It appears that these phases may have similar structures, but the poor orientation of the 35 Å reflection indicates that this reflection is not given by the well oriented phase. This could be explained by the idea that the poorly oriented phase consists of coiled DNA molecules held together in a coiled form by labile histone bridges. When this structure is stretched, the DNA molecules straighten, the bridges break, and the well oriented phase is formed.

The pattern at low humidity appears to be due mainly to histone. The pattern due to the DNA becomes diffuse and fades because the molecules crumple somewhat and are forced into contact. The histone structure collapses on dehydration and the consolidated histone gives diffuse 4·7 and 9·7 Å diffraction rings. The histone bridge spacing could still show clearly because histone is concentrated near the bridge-attachment points on the DNA. The appearance of the 75 Å spacing, twice the bridge-spacing at low humidity, can be explained if some of the bridges coalesce in pairs.

(ii) *The* in vivo *pattern.* The 38 Å ring observed *in vivo* is fairly sharp. This indicates that the structure consists of a fairly regular and somewhat extended arrangement of nucleohistone molecules. Such a structure could consist of DNA molecules linked together by histone bridges. Because it is sharp, and because the spacing does not vary much with concentration, the 38 Å reflection resembles the 35 to 37 Å meridional reflection from fibres more than the diffuse equatorial reflection. For these reasons, we think the ring corresponds to diffraction from the histone bridges and not to diffraction at right angles to DNA molecules and corresponding to an equatorial reflection. Diffraction at right angles to the DNA molecules may be weak because they are inclined to each other and not very regularly arranged. If this were so, the equatorial reflection would appear on partial drying because the molecules pack more nearly parallel.

(iii) *Relation of the fibre pattern to the* in vivo *pattern.* The highest spacing for the equatorial reflection we have observed is 35 Å. This would correspond to a concentration of 40 % nucleohistone if the molecules were in parallel hexagonal array and if the nucleohistone contained 47 % DNA. The concentration of nucleohistone in the wet chromosomes we study is between 20 and 30 %. The DNA molecules are very long and the histone is bound to them, and therefore we think it unlikely that the DNA molecules undergo radical rearrangement in the reversible transition from wet gel to

oriented partly-dried fibre. We suggest, therefore, that the structure *in vivo* and the oriented structure we study in fibres, although they have a different hydration, are basically similar.

The diffraction patterns of nucleohistone are the same whether, during preparation, it has been preserved intact, dispersed as single nucleohistone molecules in water, or dissociated into DNA and histone in strong salt solution. This shows that the three-dimensional complex of DNA and histone can be taken apart reversibly (Wilkins, Zubay & Wilson, 1959) either by separating histone from DNA at high ionic strength or by breaking the histone bridges at low ionic strength. It also supports the idea that there is only one type of nucleohistone molecule and that these molecules are linked together to form only one type of structure in unstretched nucleohistone.

5. Discussion

There are a number of pieces of circumstantial evidence suggesting that nucleohistone may be a coiled structure.

1. The 35 to 37 Å diffraction arc is never well oriented.
2. The spacing of the arc is generally greater than the observed pitch of the DNA helix.
3. We fail to obtain an oriented pattern from wet fibres.
4. The radius of gyration of nucleohistone molecules in solution is less than that of DNA, and the flow birefringence at limiting rates of shear is less (Zubay & Doty, 1959).
5. The birefringence and ultra-violet dichroism (Caspersson, 1940) of most chromosomes is low. Sperm heads that contain a histone-like protein and give an X-ray diffraction pattern like that of nucleohistone (Wilkins, 1956*a*,*b*) are not birefringent.
6. Electron microscope photographs of nuclei containing nucleohistone do not show straight parallel threads but often appear to show coiled threads of diameters between 50 and 200 Å (e.g. Ris, 1958; Kaufmann & McDonald, 1956),
7. In the light microscope, mitotic chromosomes are seen to consist of coiled-coil structures (e.g. De Robertis, Nowinski & Saez, 1954).

It is clear from 3, 5 and 6 that DNA in unstretched nucleohistone is not well oriented. In contrast, X-ray diffraction (Feughelman *et al.*, 1955) and optical studies (Schmidt, 1937; Caspersson, 1940) of nucleoprotamine show that it contains well oriented DNA and the electron microscope (e.g. Gall & Bjork, 1958) shows oriented fibres or sheets in immature sperm heads that probably contain protamine (Dass & Ris, 1958).

The evidence suggests that combination of histone with DNA causes DNA molecules to coil and to be joined together by histone bridges. The function of these bridges may be to link together genetically similar DNA molecules to form a polytenic chromosome which is compact and transportable during mitosis. Histone has not been found in bacteria (Zubay & Watson, 1959; Wilkins & Zubay, 1959) or in bacteriophage (Hershey, 1955). Possibly histone is not required to link DNA molecules in micro-organisms because the number of molecules is smaller than in higher organisms.

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J. Mol. Biol. (1959) **1**, 186-187

Letters to the Editor

The Transition between Straight and Coiled-Coil Configurations of the α-Helix

The outstanding features of the usual α-pattern in keratin-myosin-epidermin-fibrin proteins are a 5·2 Å meridional spacing and an equatorial spacing of approximately 9·5 Å. These features are believed to be due to the α-helix structure in a coiled-coil form (Crick, 1953; Pauling & Corey, 1953). Rudall (1950, 1952) has described a transition between this α-pattern and a cross-beta (Xβ) pattern (Astbury *et al.*, 1935). The Xβ pattern also has two prominent spacings but in somewhat different positions: the meridional spacing occurs at 4·65 to 4·75 Å and the equatorial spacing is 0·2 to 0·6 Å greater than that observed in the corresponding α-pattern. (An X-ray diffraction pattern observed in flagella has been called a cross-beta pattern but the main spacing on the equator occurs at about 5 Å rather than 10 Å (Parker & Rudall, 1957).) In addition the orientation of the protein, as indicated by the length of the diffraction arcs, is distinctly lower although the infra-red dichroism is qualitatively the same as that for the α-form. The two main conditions for the conversion of the α-protein into the cross-beta structure are a high temperature (above 65°C) and swelling in water. The fibres are thermally contracted and when stretched and dried produce the desired Xβ X-ray pattern. However, stretching from a contracted state is not essential: oriented fibers held at constant length and heated in water show the transformation from α to Xβ. The original explanation of the cross-beta pattern, as the name implies, was in terms of a beta-protein structure with the extended polypeptide chains oriented perpendicular to the fiber axis rather than parallel to it as in the ordinary beta structure: but it is difficult to see what might cause the extended polypeptide chain to orient in this direction.

Although we cannot hope to establish the correctness of a structure on the basis of two spacings only, the cross-beta pattern can be accounted for by a structure consisting of the α-helix and we believe this to be the only exactly defined explanation. Specifically we would ascribe the transition to the Xβ structure to a loss in supercoiling of the α-helix. This would relieve the strain energy of the helix backbone although it might result in less efficient packing of the side chains. In the Xβ structure it is suggested that the helices pack parallel or antiparallel to one another in small microcrystals with adjacent microcrystals packing at an average angle of 20°. Presumably the heating and swelling in water, which are essential factors in making this transition, provide the molecules with sufficient freedom and thermal agitation to perform the necessary untwisting and to re-adjust to the new packing arrangement. In this structure the meridional reflection in the X-ray diffraction pattern would be due to the first row-line spots on the 5·4 Å layer-line being sufficiently disoriented so as to sweep out an arc with an almost even intensity across the meridian (see Fig. 1). Since the equatorial spacing on the first row line (which is due to the average distance between helices) occurs at 10·1 Å, it may be calculated that the main reflection on the layer-line should occur at 4·7 Å; this reflection would appear to be meridional. This is the value observed in the cross-beta pattern. The long arcs on both the equator and the meridian, and the lack of a pronounced maximum in intensity on the meridian

in the Xβ pattern, are consistent with the large disorientation factor necessary to produce this reflection across the meridian from the two off-meridional diffraction spots.

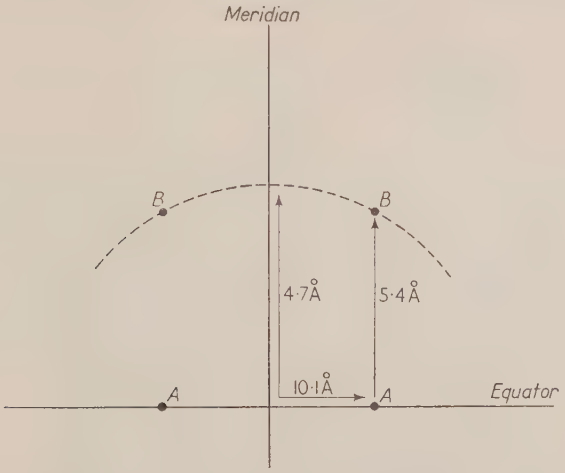

Fig. 1. Diagram showing the position of the two most intense spots on the X-ray diffraction pattern of an α-helix: A—the first row equatorial spot, B—the first row and 5·4 Å. layer-line spot. If disorientation were great enough, the spots B, B would sweep out the arc shown by the dashed line which would lead to a high intensity on the meridian with a spacing of 4·7 Å.

An obvious corollary to the suggestion made here is that a system of closely packed α-helices should give an X-ray powder diffraction diagram with two principal rings: if the inner ring associated with the side-chain spacing occurs at 10·1 Å we would expect the outer ring to appear at 4·7 Å. It is interesting to note that most globular proteins give such a powder pattern in their native state (Low, 1953) and it is possible that the main periodicities within the globular protein molecules are roughly the same as in the cross-beta structure. However, it is not likely that groups of more than three or four helices occur closely packed together because of the small size of globular proteins. Others have suggested that the powder diffraction pattern of globular proteins might be due to an underlying α-helix structure (Pauling & Corey, 1951; Arndt & Riley, 1955). The intention here is to emphasize the precise values of the two main spacings and the similarity to the cross-beta pattern.

I am indebted to my colleagues here and especially Dr. M. H. F. Wilkins for help they have given me in formulating these ideas.

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"Alkaline" Polyadenylic Acid

The X-ray diffraction pattern given by dry fiber bundles of polyadenylic acid drawn from a solution of pH 7 or 8 and suitably stretched is distinct from that given by fibers drawn from solutions of pH 5 or 6 (Rich, 1957; Watson, 1957; Morgan & Bear, 1958), and is characterized by a well-oriented meridional reflection of moderate intensity with a Bragg spacing of 5·7 Å. The 11 reflections of this pattern so far registered (see Plate I) have been indexed on the basis of a monoclinic cell 12·0 × 5·7 × 15·3 Å with $\beta = 83{\cdot}5°$, as shown in the following table:

hkl	intensity	*d*, observed	*d*, calculated
001	v.s.	15·2 ± 0·5 Å	15·2Å
002	v.w.	7·9 ± 0·2	7·60
100	w.	11·9 ± 0·3	11·9
200	m.	5·8 ± 0·2	5·95
101	w.	9·9 ± 0·2	9·95
202	w.	5·0 ± 0·1	4·98
203	v.w.	4·3 ± 0·1	4·10
010	v.s.	5·7 ± 0·2	5·70
213	s.	3·4 ± 0·1	3·27
012	v.w.	4·5 ± 0·1	4·56
020	m.	2·9 ± 0·1	2·85

To obtain this oriented pattern it was sufficient to stretch the fibres in a bath of 80 % ethanol at room temperature and to maintain the tension whilst the bundles dried after removal of the bath. If the tension was relaxed, an amorphous pattern resulted. If, after stretching and whilst tension was maintained, the bath was changed to 65 % ethanol and its pH was lowered to 4 with HCl, the earlier known pattern with intense spacing of 3·8 Å was seen but with all orientation lost. If, however, the bath was acidified to pH 4 while the ethanol concentration was higher than 65 %, the fiber bundles continued to give the new diffraction pattern. These observations are interpreted as indicating that there exists no simple steric relation between the two polyadenylic acid configurations, but rather that the "alkaline" configuration must essentially be dissolved before the "acid" one can form.

The density of fibre bundles giving the alkaline pattern, measured by flotation in mixtures of C_6H_6 and $CHCl_3$, was approximately 1·45. There are thus about 2·6 molecules of $(C_{10}H_{11}O_6N_5P)^- Na^+$ per cell. It seems probable, in view of the lack of trigonal symmetry, that the true number of monomer units per cell may be 4, a high proportion of amorphous polymer accounting for the low density observed. There is no indication that the space group is other than $p1$.

No further work on the pattern is contemplated.

PLATE I. X-ray diffraction pattern from fibre bundle of sodium polyadenylate, stretched to 1·5 times original length in 80 % ethanol and dried under tension. CuKα radiation, helium-filled camera, 5·1 cm specimen to film distance, 18 hours exposure. Flat film, fibre axis vertical.

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We wish to thank Dr. Roland F. Beers for his generous gift of polymer and Dr. Elkan R. Blout for providing facilities for X-ray diffraction. The work was supported by grant CY 3335 from the U.S. Public Health Service.

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Nuclear Magnetic Relaxation of Water Protons by Ferrihemoglobin and Ferrimyoglobin

Paramagnetic ions are effective in inducing spin-lattice (T_1) nuclear magnetic relaxation of the protons in an aqueous solution. Under suitable conditions, the relaxation effect is proportional to $\mu_p^2 \mu_{ion}^2 c/aD$, where μ_p and μ_{ion} are the magnetic moments of proton and ion, a is their distance of closest approach, c is the concentration of paramagnetic ions, and D is the coefficient of relative diffusion for water molecules and the ion. One of us has previously reported that there is a perceptible, but relatively small, relaxing effect of ferrihemoglobin (Hb^+) on water protons (Davidson & Gold, 1957). This was interpreted as indicating that the heme groups are not on the surface of the protein. We have now carried out further measurements on Hb^+ and ferrimyoglobin (Mb^+) which show that the heme groups in myoglobin are much more effective in relaxing water protons.

The measurements were made by the Bloch rapid-passage method, using the detailed technique described before (King & Davidson, 1958), with a Varian high resolution machine at 60 Mc/s ($H = 14{,}100$ gauss). Sperm-whale ferrimyoglobin crystals as a paste precipitated from 1 : 1 K_2HPO_4/NaH_2PO_4 were kindly supplied by Dr. J. C. Kendrew. Human ferrihemoglobin was prepared by air oxidation of HbO_2 at pH 5, and also by ferricyanide oxidation of HbO_2 or HbCO at pH 6·8. Measurements were made with solutions at pH 6·8–7·0, and concentrations were determined by spectrophotometry. The solvent was a mixture of H_2O and D_2O (usually 1 : 1) to decrease the contribution to the relaxation by the proton-proton interaction. As a control, the solutions were converted to ferrihemoglobin cyanide and ferrimyoglobin cyanide (HbCN and MbCN). The relaxing effect of the heme-cyanide group is very much less than that of the ferriheme group.

The results for one experiment are given in detail: Mb^+, $c = 5{\cdot}3 \times 10^{-4}$ mole liter^{-1}, $T_1 = 0{\cdot}98$ sec; MbCN, $c = 5{\cdot}3 \times 10^{-4}$ mole liter^{-1}, $T_1 = 4{\cdot}42$ sec,

$$[(1/T_1)_{Mb} - (1/T_1)_{MbCN}]/c = 1500 \text{ sec}^{-1} \text{ mole}^{-1} \text{ liter}.$$

About one-third of the effect in the MbCN solution is due to the protein and the rest to the solvent.

The overall results so far are: for Mb^+, $1/T_1 = 1400\ (\pm 100)\ c$; for Hb^+, $1/T_1 = 250\ (\pm 50)\ c$ (concentration expressed in heme groups per liter); for Fe^{+++}, $1/T_1 = 1{\cdot}15\ (\pm 0{\cdot}1) \times 10^4\ c$. The Hb^+ result is greater than that reported previously ($1/T_1 = 110\ c$), presumably because of the improved measurement technique. Similar results, as regards the comparison of Hb^+ and Mb^+, have been obtained by Bovey, Matsumiya & Lumry (private communication).

Nuclear magnetic relaxation by paramagnetic ions is a rather complicated phenomenon. In addition to the factors contained in the expression in the first paragraph, it may depend on the electronic structure of the paramagnetic species, and especially on the electron paramagnetic relaxation time. (Thus ferrohemoglobin with a Bohr magneton number of 5·46 (Pauling & Coryell, 1936; Coryell *et al.*, 1937) is much less than $(5{\cdot}46/5{\cdot}80)^2$ as effective as ferrihemoglobin because the former has a much

shorter paramagnetic relaxation time.) These electronic factors however are constant in the ferrimyoglobin-ferrihemoglobin comparison. The significant conclusion of the present work is then that the heme group in hemoglobin is more effectively shielded from the solvent than is the heme group in myoglobin.

The low resolution, three-dimensional crystal structure of myoglobin shows the heme group essentially on the surface of the protein (Kendrew *et al.*, 1958), with the dense cores of several of the polypeptide chains running around the edge of the heme group. The X-ray data do not yet reveal the extent to which the side chains shield the solvent from the iron ion. The present experiment cannot be given a very quantitative interpretation, but the fact that Mb^{+} is about one-sixth as effective as Fe^{+++} itself suggests that the side chain shielding is not very great.

In hemoglobin, the heme group is more effectively shielded from the solvent by the protein. The heme group may be in a crevice but we cannot say at present whether the relaxation is mainly due to a direct magnetic interaction with outer water or whether it is due to a magnetic interaction with crevice water, followed by diffusion exchange of crevice and outer water.

Finally, it may be recalled that myoglobin (Lein & Pauling, 1956), as well as hemoglobin (St. George & Pauling, 1951), binds ethyl *iso*cyanide more strongly than *t*-butyl *iso*cyanide. For the case of myoglobin at least, this is probably due to steric hindrance by the side chains alluded to above, but it does not prove that the heme group is buried beneath the surface of the protein.

Work on these and related problems is continuing. We are indebted to Dr. J. C. Kendrew, Dr. J. Vinograd, and Mr. W. Hutchinson for their advice and help.

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Structure and Dimensions of Bacteriophage ϕX174 from Electron Microscopy

We have prepared samples of bacteriophage ϕX174 (Sinsheimer, 1959; Tessman, 1959) for electron microscopy by the following procedure. The phage, obtained originally from Dr. R. L. Sinsheimer, was grown on *Escherichia coli* strain C, in synthetic medium buffered with tris-HCl at pH 7·4. The lysed culture was centrifuged at low speed to remove cell debris. After making the supernatant 3 M in ammonium sulfate, the phage was precipitated by centrifuging at 15,000 rev/min. The pellet was resuspended in 0·01 M-ammonium acetate, pH 7·4, and adsorbed on a diethylaminoethylcellulose anion exchange column. The phage was eluted from the column by 0·10 M-ammonium acetate. The purification of larger quantities of virus has subsequently been carried out by modifications of the above procedure.

Although ϕX174 is large enough to be readily visible by the techniques of conventional electron microscopy, our specimens were prepared by a method developed for the observation of much smaller particles. The phage were supported on the surface of freshly cleaved mica, and shadowcast with platinum which was later stripped from the mica (Hall, 1956). A typical micrograph is shown in Plate I (a) where several characteristic features are evident: (i) each virus has a high spot in the center; (ii) the shadows are polygonal and sometimes a sharp spike may be discerned corresponding to the high central point; (iii) the periphery of the virus is seen to consist of granules or "knobs" arranged as at the corners of a polyhedron, but the shadowing angle (shadow-to-height ratio 2·75 : 1) is such that the fine structure is obscured on the side facing the evaporating source.

In Plate I (b), at higher magnification and at a shadow-to-height ratio of about 1 : 1, the fine structure is better resolved although there is the illusion that the particles are flattened. Here one sees that the central high point is surrounded by five granules arranged with pentagonal symmetry. On the side of the virus facing the evaporation source a lower tier of granules also intercepts metal which tends to fill in the space between the members of the upper five. The presence of a second lower tier may also be inferred from the fact that the height of the upper tier, as deduced from shadow lengths, is about equal to the distance along the two granules forming the side of the pentagonal pattern. Except for a few particles which appear to have disintegrated and those that are in contact with each other, all particles present the same aspect, when properly shadowed, as illustrated in Plate I (b). We have seen no evidence for tails, attached or detached.

A micrograph of a specimen prepared by a negative staining technique (Horne & Brenner, 1958; Hall, 1955) is illustrated in Plate I (c). In such micrographs the virus outlines are polyhedral. We have also made micrographs of a purified sample of ϕX174 obtained directly from Dr. R. L. Sinsheimer and have observed the same morphological features described above both by shadow casting and negative staining techniques.

Measurements of particle diameters (assuming a roughly spherical shape) from

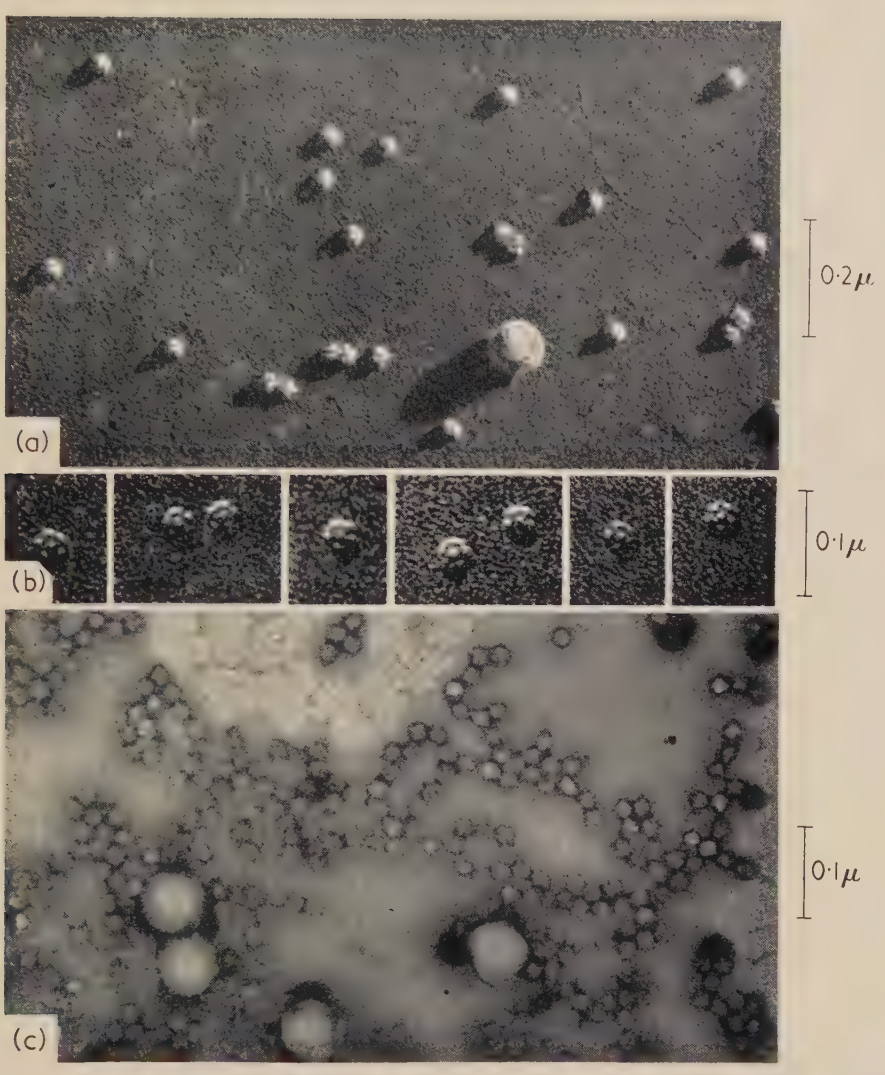

PLATE I (a). Bacteriophage ϕX174 on a mica substrate shadowed with platinum at a shadow-to-height ratio of about 3 : 1. Magnification 106,000 ×.

(b) ϕX174 shadowed at an angle 1 : 1 shown at various azimuths with respect to the direction of shadowing. Magnification 190,000 ×.

(c) ϕX174 dried down in neutral phosphotungstic acid. Magnification 164,000 ×.

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shadowed specimens may be made in two ways: (a) measurement of apparent particle diameter perpendicular to the direction of shadowing, including the cap of metal, and (b) measurement of shadow lengths and calculation of particle heights from shadow-to-height ratios obtained from polystyrene spheres in the same field. Results of an unpublished study by one of us (C.E.H.) indicate that under the standardized conditions used the apparent diameters of approximately spherical particles including the cap of metal are too large by 50 to 75 Å, while the shadow lengths are a good measure of particle diameter.

Measured diameters of shadowed ϕX174 are about 50 Å greater than heights which would be expected for a spherical particle, indicating that there is negligible flattening due to drying forces. The average of all determinations from shadow lengths and from diameters minus 50 Å for the metal, of the M.I.T. virus and that obtained from Dr. Sinsheimer, is 248 Å with a mean deviation of $\pm$ 10 Å. Averages for the diameters from the two sources are 243 Å and 252 Å respectively which are within experimental error of the overall mean. Diameters from negatively stained preparations are slightly smaller than from shadowed preparations but in these micrographs there is some difficulty in locating the particle edges accurately.

If a sphere were to contain the amount of protein and deoxyribonucleic acid (DNA) that has been determined for ϕX174 by Sinsheimer (1959) ($4{\cdot}6 \times 10^6$ mol wt units of protein of density 1·3 and $1{\cdot}7 \times 10^6$ mol wt units of DNA of density 1·7) the sphere would have a diameter of 240 Å, in good agreement with the electron microscope results.

The regular structural pattern of the shadowed phage, as well as the polygonal outline of the shadows and of the negatively stained particles, strongly suggests that the structure of the phage is related to a regular polyhedron. Williams & Smith (1958) have demonstrated that *Tipula* Iridescent virus, of approximately 1300 Å diameter, has the shape of an icosahedron with faces that are planar rather than granular as with ϕX174. Angularities in outline of small viruses have also been noted by Williams (1954) and Kaesberg (1956), and Sinsheimer (1959) has noted occasional evidence for a polyhedral shape in micrographs of ϕX174.

A likely interpretation of our pictures of ϕX174 is that the phage contains a shell of twelve "knobs" arranged regularly as on the faces of a regular dodecahedron. This is based on the observation of one apical and five surrounding peripheral knobs, with the suggestion of a second underlying tier similarly arranged. This interpretation implies that every phage in the shadowed pictures would have to rest on the knob opposite the central apex seen in every particle. It is possible that upon drying there would be some distortion of the phage that would provide a broader base of contact. The alternative explanation that the phage is not regular but contains a broad face appears unlikely because such a particle would be expected to present a variety of appearances in shadow-cast micrographs, unless the broad face preferentially adsorbs to the substrate. If ϕX174 does contain twelve regularly arranged knobs one might expect that the phage could sometimes orient itself with three knobs in contact with the substrate. This would produce both a narrower shadow near the base and a pattern of highlights which we do not observe. The outline of negatively-stained virus is consistent with the outlines of the type of polyhedron postulated although the resolution is not adequate to establish the shape unequivocally.

Crick & Watson (1956) have speculated that many small viruses may be made up of subunits, related by symmetry elements. The electron microscope pictures give suggestive evidence that this is true for ϕX174. In particular, twelve regularly

arranged knobs would be related at least by four three-fold and three two-fold rotation axes of symmetry. The pictures are consistent with the existence of five-fold symmetry axes, as well, but this would require that each knob contained a multiple of five symmetrically arranged subunits, about which we have no evidence. We believe these pictures confirm the suggestion of common structural features of small viruses, whether plant, animal or bacterial.

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1. The *Journal of Molecular Biology* will publish papers on the nature, production and replication of biological structure at the molecular level, and its relation to function. Suitable subjects are sub-cellular organization: molecular genetics: structure and replication of viruses: molecular structure of muscle, nerve and other tissues: structure of proteins, nucleic acids, carbohydrates, lipids, etc., and their synthetic analogues, as investigated by X-rays, light absorption and other methods: problems of inter- and intra-molecular energy transfer.

2. The Journal will also publish Letters to the Editor (averaging 1000 words), and occasional review articles dealing with a more restricted or immediately topical field than those in review journals. (The Editor should be consulted in advance by anyone who considers submitting such a review.)

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Papers should contain the maximum density of information compatible with clarity, and every sentence should be scrutinized to ensure that its presence is essential. Authors should include enough experimental details to enable others to repeat the work (these may be printed in small type). Careful preparation of manuscript will aid prompt publication; carelessness will cause delay.

4. *Submission of Manuscripts.* Manuscripts should be sent to Dr. J. C. Kendrew, Peterhouse, Cambridge, England, or to any member of the Editorial Board. The submission of manuscript will be taken to imply that the material is original, and that no similar paper has been or will be submitted for publication elsewhere.

Papers will normally be published in English. Manuscripts in French or German will also be accepted, though the use of these languages may cause delay in publication.

5. *Copy.* Papers should be typewritten in double spacing *throughout* (including tables, legends and footnotes), on one side of quarto paper, with a margin of 1 inch all round. The position of tables and illustrations should be indicated in the text; tables and legends for illustrations should be typed separately at the end of the manuscript. All pages should be numbered serially and securely fastened together. Two copies of each manuscript should be submitted, together with three copies of the summary (see § 6) and four copies of the abstract (see § 9).

A short running title of not more than 40 characters (including spaces), suitable for page headings, should be indicated if the full title is longer than this.

The name of the laboratory where the work has been done should be indicated on the title page. All typescript (including the references) should be carefully checked for errors.

6. *Summary.* A summary will be printed at the head of all papers (excluding Letters to the Editor); this should generally not exceed 300 words, and should be intelligible to the general reader without reference to the main text. Abbreviations should be avoided. Summaries of papers to be printed in French or German will be in English, and should be longer (up to 1000 words) for the benefit of readers not familiar with these languages.

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